

BIOLOGICAL CONTROL POTENTIAL OF *PASTEURIA PENETRANS*

By

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To Yanping and Min.

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BIOLOGICAL CONTROL POTENTIAL OF *PASTEURIA PENETRANS*

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Biological control offers an alternative or supplemental management tactic to chemical or cultural control of plant-parasitic nematodes. One biological control agent that has shown great potential for suppressing field populations of many plant-parasitic nematodes is *Pasteuria* spp. This microorganism is a mycelial, endospore-forming, bacterial parasite of root-knot nematodes. One species, *Pasteuria penetrans*, which is reported to be specific for root-knot nematodes, *Meloidogyne* spp., was chosen for study of its biological control potential against *Meloidogyne arenaria* on peanut. The objectives were to quantify the densities of endospores in tomato root material, to determine levels of endospores required to control *M. arenaria* on peanut, to determine the quantitative suppression of *M. arenaria* on peanut by *P. penetrans*, to estimate incidence of attachment of endospores to *Meloidogyne* spp. using tally threshold, and to study the ultrastructure, morphology, and sporogenesis of four isolates of *P. penetrans*. Mortar disruption and machine disruption were the most accurate methods for quantification of endospores in tomato root material. When endospores were inoculated in microplots at 0, 1,000, 3,000, 10,000, and 100,000 endospores/g of soil, root galls and pod galls were reduced at 10,000 and 100,000 endospores/g of soil. The quantitative suppression of *M. arenaria* on peanut by *P.*

penetrans was evaluated using a 6×6 factorial experiment in field microplots over 2 years. Numbers of eggs per root system, juveniles per 100 cm^3 of soil at harvest, root and pod galls decreased with increasing *P. penetrans* levels and increased with increasing nematode inoculum levels. When *P. penetrans* levels was increased by 10%, the number of eggs per root system, root galls, pod galls, and juveniles per 100 cm^3 of soil at harvest decreased by 8.6%, 7.8%, 8.4%, and 8.8%, respectively. Estimating numbers of endospores attached per juvenile using various tally thresholds can reduce the work load of counting every endospore on a juvenile. Electron microscopy studies showed that the sporogenesis of *P. penetrans* was similar to that of other gram positive bacteria, especially the seven stage scheme reported for *Bacillus thuringiensis*.

Chapter 1 INTRODUCTION

Peanut Root-Knot Nematode

Biology

Meloidogyne arenaria is a sedentary endoparasite. The developmental stages include egg, four juvenile stages, male, and female. Following embryogenesis, the first stage juvenile molts to form a second-stage juvenile (J2) within the eggs. The second-stage juvenile hatches and becomes the infective stage. The hatched juveniles move through the soil pores in a film of water surrounding soil particles. They penetrate host roots, generally well behind the zone of differentiation. After penetration, the nematodes establish a feeding site (giant cells) in vascular tissue. As a result of nematode feeding, cells of the roots, pegs, and pods increase in size and number, forming galls, knots, and warts. The nematode molts three more times, developing the third, fourth, and the adult stages. The males are vermiform, whereas the females are white and globose-pyriform shaped. Generally, 200 to 1,500 eggs are laid in a gelatinous matrix. Egg masses of *M. arenaria* race 1 may exist in large numbers on pegs, pods, and roots during the growing season, but decrease dramatically after peanut harvest. Juveniles exist throughout the year and are the major survival stage.

Distribution and Importance

The peanut root-knot nematode, *Meloidogyne arenaria* (Neal) Chitwood, is the most widespread and destructive nematode pest of peanut (Minton and Baujard, 1990; Porter et al., 1984). There are two host races (types) of this pest, which can be distinguished only on the basis of their reaction on peanut. Race 1 infects and reproduces on peanut, whereas race 2 does not. Both races are widespread in many areas where peanut is produced, and they both cause diseases on other agronomic, vegetable, ornamental, and fruit crops. The presence of two races makes the use of routine nematode advisory services difficult because of the time required to determine the race identity.

Meloidogyne arenaria has been reported on peanut in China, Egypt, India, Israel, the United States, and Zimbabwe (Minton and Baujard, 1990). In the United States, race 1 of the peanut root-knot nematode is common in peanut fields in Alabama, Arkansas, Florida, Georgia, and Texas. Sporadic occurrences have been reported in North Carolina, South Carolina, and Virginia. Numerous juveniles (infective stage) are generally distributed throughout the soil profile of infested fields. In fact, in deep, sandy soils, the largest numbers of the infective stage are generally found 30 to 123 cm deep at planting (Dickson and Hewlett, 1988b). The juveniles are mobile and can move up from the deeper soil depths to infect plant roots, but hard pans or soils with underlying clay restrict the downward movement of the nematode.

Meloidogyne arenaria is the predominant, aggressive pathogen on peanut and causes substantial yield losses at relatively low population densities. In Texas, initial population densities of 9 to 19 J2/100 cm³ of soil were sufficient to cause 10% suppression of yield (Wheeler and Starr, 1987), whereas the damage threshold in Florida was determined to be as low as 1 J2/100 cm³ of soil (McSorley et al., 1992).

Symptoms and Diagnosis

Peanut plants in soils infested with peanut root-knot nematode generally have noticeable above- and below-ground symptoms. Definite above-ground symptoms normally appear 75 to 90 days after planting but may appear sooner, depending on the initial population density of nematodes in the soil and on environmental conditions (Minton and Baujard, 1990). Root-knot nematodes are spotty in distribution, and infected plants have variable symptoms. Plants that are heavily infected are stunted and yellow, and pod production is reduced. If drought occurs near the end of the season, it may greatly increase the severity of root-knot, and the weakened plants may die.

To diagnose root-knot, the roots, pegs, and pods should be examined for the presence of nematodes (Minton and Baujard, 1990; Porter et al., 1984). Juveniles, which are too small to be seen with the unaided eye, infect peanut roots soon after planting. The characteristic symptom of the disease is the abnormal swelling (galls or knots) on the roots, pegs, and pods. Galls and egg masses form rapidly and become apparent on the roots 55 to 90 days after planting. The galls on peanut roots are small and generally discrete, whereas galls on other host crops, such as tomato, squash, and tobacco, may be large and coalesced. Nematode galls can be distinguished from nodules formed by *Bradyrhizobium* spp.; the latter are distinct, round swellings that appear to be attached to the root and are easily detached. Nematode galls are irregularly shaped swellings that constitute a part of the root and cannot be detached without destroying the integrity of the root. The nematode also may infect and reproduce in the rhizobium nodules (Minton and Baujard, 1990; Porter et al., 1984).

Soon after blooming and the initiation of pod set (about 45 days after planting), the nematode may infect pegs and pods. Early infection may result in a weakened peg and an aborted pod, or if a pod forms it may be detached from the plant and remain in the soil at harvest.

Shells of pods that set may become heavily infected and extensively galled late in the season, resulting in yield losses. Galled tissue is especially vulnerable to infection by several soilborne fungi, which greatly increases the root deterioration.

Management

Currently there are no peanut cultivars with resistance to *M. arenaria* race 1 (Minton and Baujard, 1990; Porter et al., 1984). Consequently, producers must rely on cultural practices, such as crop rotation, winter cover crops, and crop destruction, and the application of nematicides for management of nematodes.

Pasteuria penetrans

Historical Background

Pasteuria Metchnikoff, 1888, was first described as a bacterial parasite of water fleas, *Daphnia magna* Straus. Metchnikoff (1888) named the bacterium *Pasteuria ramosa* and stated “*Pasteuria* sp. was able to undergo as many as five longitudinal divisions at the same time, giving it a characteristic fan shape.” He also tried to culture the bacterium, but was unsuccessful.

Over the years, Metchnikoff’s paper with its concept of longitudinal division and accompanying drawings showing “stalked” spores intrigued investigators. The bacterium, however, was not found again and consequently Metchnikoff’s work was considered erroneous (Hirsh, 1972; Migula, 1900). Little additional information was published on the bacterial parasite of water fleas until Sayre and Wergin (1977), who studied the ultrastructure of a bacterial parasite of root-knot nematodes, drew attention to its resemblance to *Pasteuria ramosa* (Judicial Commission of the International Committee on Systematic Bacteriology, 1986). They

subsequently rediscovered *P. ramosa* infecting *Moina rectirostris* Leydig, a member of the Daphnidae (Starr et al., 1977). Thus, the genus *Pasteuria* as described by Metchnikoff is conserved (Starr et al., 1983; Judicial Commission of the International Committee on Systematic Bacteriology, 1986), but the research emphasis shifted to species of *Pasteuria* that parasitized plant-parasitic nematodes.

Cobb (1906) was the first to report an organism resembling *Pasteuria* sp. infecting a nematode, *Dorylaimus bulbiferous*. He erroneously viewed these spores as “perhaps monads” of a parasitic sporozoan. This placement persisted for nearly 70 years. A more precise but still incorrect placement, *Duboscqia* Perez 1908, was suggested by Micoletzky (1925). Later, Thorne (1940) described in detail a parasite from *Pratylenchus pratensis* (de Man) Filipjev and, on the assumption that the organism was similar to the nematode parasite described by Micoletzky, named it *Duboscqia penetrans*. It was not until the nematode parasite was examined under the electron microscope that its affinities with bacteria rather than protozoa were observed, and it was then named *Bacillus penetrans* by Mankau (Esser and Sobers, 1964). Sayre and Starr (Kerry, 1987) drew attention to the fact that *B. penetrans* resembled the actinomycete *Pasteuria ramosa* (Sayre et al., 1983) and subsequently renamed the organism *Pasteuria penetrans*.

Members of *Pasteuria*

Pasteuria species are gram-positive, dichotomously branched, endospore-forming bacteria with septate mycelium. The terminal hyphae of the mycelium elongate to form sporangia and eventually endospores. The primary colonies are shaped like cauliflower florets or elongated grapes in clusters. Daughter colonies are formed by fragmentation of mother colonies, and the daughter colonies in turn produce doublets and quartets of sporangia, and finally the single sporangium containing a mature endospore. Endospores are nonmotile and

resistant to desiccation and elevated temperatures. The organisms are endoparasites of freshwater, plant-parasitic, and soil invertebrates.

Members of *Pasteuria* have not been grown in pure culture; they are cultured on a nematode or water flea host. *Pasteuria* spp. are differentiated by host specificity, developmental characteristics, and size and shape of sporangia and endospores (Sayre and Starr, 1989). Four species of *Pasteuria* have been described. *Pasteuria ramosa*, which parasitizes water fleas of the genera *Daphnia* and *Moina*, is the type species of the genus. The other three species of *Pasteuria* are parasites of plant-parasitic nematodes: *P. penetrans* on *Meloidogyne* spp., *P. thornei* on *Pratylenchus* spp., and *P. nishizawae* on cyst nematodes of the genera *Heterodera* and *Globodera*. Two other proposed new species of *Pasteuria* have been isolated from *Heterodera goettingiana* Liebscher in Münster, Germany (Sturhan et al., 1994), and from *Belonolaimus longicaudatus* Rau in Florida (Giblin-Davis et al., 1995).

There is still considerable confusion about the taxonomy of *Pasteuria*. The confusion arises from the criteria used for differentiating the species. The criteria, once seeming explicit, are challenged by new isolates of *Pasteuria* spp. from plant and soil nematodes. Host specificity is overlapped in many isolates of *Pasteuria* spp. Sizes of endospores and sporangia are correlated with host nematode genera (Ciancio, 1995). Diameter of sporangia varies from $< 2 \mu\text{m}$ for an isolate from *Criconebella* sp. in Florida (pers. obs.) to $8 \mu\text{m}$ for an isolate from *Axonchium valvulatum* in Sri Lanka (Ciancio et al., 1994). Shapes of endospores and sporangia are different among various isolates obtained from different nematode genera.

Some newly reported isolates of *Pasteuria* spp. display a cross-genera host range and various biological and ecological characters. Isolates of *P. penetrans* reported from China (Pan et al., 1993), Puerto Rico (Vargas and Acosta, 1990), and the United States (Mankau, 1975; Oostendorp et al., 1990) parasitize both *Meloidogyne* spp. and *Pratylenchus* spp. An isolate reported from India parasitizes both *Heterodera* spp. and *M. incognita* (Bhattacharya and

Swarup, 1988), whereas another Indian isolate parasitizes *Globodera* spp., *Heterodera* spp., and *Rotylenchulus reniformis* (Sharma and Davies, 1996).

Some isolates of *Pasteuria* spp. selectively parasitize various developmental stages of nematodes (Davies et al., 1990; Abrantes and Vovlas, 1988; Noel and Stanger, 1994). Davies et al. (1990) reported a *Pasteuria* sp. isolate completing its life cycle in J2 of *Heterodera avenae*, but not in females and cysts. Abrantes and Vovlas (1988) reported an isolate of *Pasteuria* sp. parasitizing juveniles and males of *Meloidogyne* sp. and juveniles of *Heterodera fici*. An Illinois isolate of *Pasteuria* sp. infected both J2 and males of *Heterodera glycines* but not females (Noel and Stanger, 1994).

Pasteuria penetrans has been reported to develop mature endospores only in females of *Meloidogyne* spp. (Sayre and Starr, 1989). An isolate of *Pasteuria* sp., however, has been reported developing mature endospores in juveniles, males, and females of *M. acronea* (Page and Bridge, 1985). Endospore-filled J2 of *Meloidogyne* sp. also were observed from a nematode suppressive soil that was infested by *P. penetrans* in Florida (Dickson, pers. comm.). One isolate of *Pasteuria* sp. was reported to complete its life cycle in J2 of *Meloidogyne* spp. on turfgrass in Florida (Giblin-Davis et al., 1990).

Several genera of nematodes have been observed to be parasitized by the bacterium at the same site. Second-stage juveniles of *Heterodera avenae*, and juveniles and adults of *Pratylenchus* sp. and *Tylenchorhynchus* sp., were infected with *Pasteuria* spp. in a nematode-suppressive soil in England (Davies et al., 1990). Although the endospores from the three nematode hosts were the same size, it is not clear if the endospore populations belonged to a single or multiple species of *Pasteuria*. The endospores obtained from J2 of *H. avenae* attached to J2 of *H. schachtii*, *H. glycines*, *Globodera rostochiensis*, *G. pallida*, and *M. javanica*, but the infection of females was not observed (Davies et al., 1990). Similarly, juveniles and adults of *Pratylenchus* sp., *Helicotylenchus* sp., and *Aphelenchoides* sp. were observed filled with

endospores of a *Pasteuria* sp. at an experimental site that was inoculated previously with *P. penetrans* endospores (Chapter 3; per. obs.). A survey of sugarcane fields in South Africa revealed that endospores of *Pasteuria* spp. attached to species of *Pratylenchus*, *Helicotylenchus*, *Scutellonema*, and to several other common nematodes (Spaull, 1981). Small endospores ($2.9 - 4.4 \times 1 - 2 \mu\text{m}$) from *P. zaeae*, *H. dihystrera*, and J2 of *M. incognita* were assumed to be *P. penetrans*, and larger endospores ($4.3 - 6.6 \times 2.0 \mu\text{m}$) from *Scutellonema* sp. and *Xiphinema* sp. were considered to be another isolate of *P. penetrans*. In a survey of plant-parasitic nematodes on turfgrass in south Florida, Giblin-Davis et al. (1990) observed that the following groups of nematodes were parasitized by *Pasteuria* spp. at various sites: *Belonolaimus longicaudatus*, *Meloidogyne* spp., and *Helicotylenchus microlobus* in Collier County; *B. longicaudatus*, *Hoplolaimus galeatus*, *Tylenchorhynchus annulatus*, and *Meloidogyne* spp. in Broward County; and *H. microlobus* and *Meloidogyne* spp. in Palm Beach County. They also reported large-endospore and small-endospore isolates parasitizing both *B. longicaudatus* and *H. galeatus* in Broward County, Florida (Giblin-Davis et al., 1990).

The current confusion in taxonomy of *Pasteuria* probably will not be clarified until the bacterial genome properties, such as size, base composition, and the DNA sequence similarity revealed by hybridization, are elucidated. Cultivation of the bacteria is crucial to understanding the complex biology and taxonomy of *Pasteuria*, but artificial cultivation of *Pasteuria* spp. has not been successful.

Biology of *Pasteuria penetrans*

Life Cycle

When J2 of *Meloidogyne* spp. move through soil, their cuticles become encumbered by endospores of *P. penetrans*. Germination of endospores occurs 4 to 10 days after the endospore-

encumbered nematode enters the plant root and begins to feed (Sayre and Wergin, 1977; Serracin et al., 1997) and is temperature dependent. The germ tube emerges through a central opening in the basal attachment layer of the endospore and penetrates the cuticle of the nematode. The process of penetration seems to be enzymatic (Mankau, 1975; Mankau et al., 1976). After entering the pseudocoel of the nematode, the germ tube develops into a cauliflower-like microcolony consisting of a dichotomously branched septate mycelium. Daughter colonies form when the intercalary cells in the microcolony lyse (Sayre and Starr, 1989). Due to some unknown triggers, the colony forms fragmentations; the terminal cells of the fragmentation enlarge and undergo sporogenesis. Eventually, quartets and doublets of developing sporangia predominate in the nematode body cavity and finally separate into a single sporangium containing an endospore. The mature endospores are released into soil when the plant root with its complement of parasitized root-knot nematode females decomposes.

Sporogenesis

Although the ultrastructure of mature endospores of each species of *Pasteuria* are unique, all species share the typical sequence of a gram-positive, endospore-forming bacterium (Sayre, 1993). Briefly, the sporogenous sequence includes the formation of a transverse septum within the endospore mother cell, condensation of a forespore from the anterior protoplasm, formation of multi-layered walls surrounding the forespore, formation of cortex and coat, formation of parasporal fibers and exosporium, and finally, maturation and release of endospores.

Systematics and Phylogeny

Modern bacterial systematics depends on both phenotypic and molecular biological characters. The phenotypic characters are still a premium in classification and identification of prokaryotes. In recent years, nucleic acid techniques have been used to determine bacterial

genome properties, such as size, base composition, and the DNA sequence similarity revealed by hybridization. For example, it is now commonly accepted that bacteria with DNA base compositions differing by more than 10 mol % guanine (G) plus cytosine (C) content (%GC) should not be regarded as members of the same genus and populations differing by more than 5 %GC values should not be regarded as the same species (Bull et al., 1992). A genomic method of separation of species is based on strains having $\geq 70\%$ relatedness and $\geq 5\%$ divergence of DNA; both parameters must be used (Goodfellow and O'Donnell, 1993).

Currently, endospore-forming bacteria are placed in 13 genera, which are separated based on morphological, physiological, and genetic diversity (Table 1.1). A commonly accepted rule is that bacteria with DNA base compositions differing by more than 10 %GC should not be regarded as members of the same genus (Bull et al., 1992). When this rule is applied to the values shown in Table 1.1, three genera, namely, *Bacillus*, *Clostridium*, and *Desulfotomaculum*, are heterogeneous. Because *Pasteuria* species have not been cultured axenically, their DNA base compositions remain unknown. Consequently, their relationship to other endospore-formers is unclear. Some molecular evidence indicates that *P. penetrans* is a deeply rooted member of the *Clostridium-Bacillus* line of descent, neither related to the actinomycetes nor closely related to the true endospore-formers (Berkeley and Ali, 1994).

Host Records

Comprehensive reviews of the host records of *Pasteuria*-like organisms have been reported by Sayre and Starr (1988) and Sturhan (1988). *Pasteuria*-like organisms have been reported from 196 species of soilborne nematodes belonging to 96 genera, from 51 countries on five continents and on various islands in the Atlantic, Pacific, and Indian oceans (Sayre and Starr, 1988; Sturhan, 1988). An updated host record is listed in Table 1.2. The updated

Table 1.1. Described genera of endospore-forming bacteria and their DNA base composition.^a

Genus	Mol %GC ^b
<i>Alicyclobacillus</i>	52-60
<i>Amphibacillus</i>	36-38
<i>Bacillus</i>	32-69
<i>Clostridium</i>	22-54
<i>Desulfotomaculum</i>	38-52
<i>Oscillospira</i>	- ^c
<i>Pasteuria</i>	-
<i>Sporohalobacter</i>	30-32
<i>Sporolactobacillus</i>	38-40
<i>Sporosarcina</i>	40-42
<i>Sulfobacillus</i>	54
<i>Syntrophospora</i>	38
<i>Thermoactinomyces</i>	52-55

^aFrom Berkeley and Ali, 1994.^bMol %GC = mol % guanine (G) plus cytosine (C) content.^c = No information available.

Table 1.2. Updated list of nematode hosts and geographical distribution of *Pasteuria* spp.

No.	Nematode	Location	Source
1.	<i>Achromadora micoletzkyi</i>	Germany	Sturhan, 1988
2.	<i>Acrobeloides buetschlii</i>	Germany	Sayre and Starr, 1988
3.	<i>Acrobeloides nanus</i>	Germany	Sturhan, 1988
4.	<i>Acrobeloides</i> sp.	Italy	Ciancio et al., 1994
		U.S.A., California	Ciancio and Mankau, 1989b
5.	<i>Actinca</i> sp.	Nicaragua	Sturhan, 1988
6.	<i>Aglenchus agricola</i>	Germany	Sayre and Starr, 1988
		England	Sturhan, 1988
7.	<i>Alaimus</i> sp.	Germany	Sturhan, 1988
8.	<i>Amplimerlinius globigerus</i>	Germany	Sturhan, 1988
9.	<i>Amplimerlinius icarus</i> (syn. <i>Merlinius icarus</i>)	Belgium	Sturhan, 1988
		Germany	Sayre and Starr, 1988
10.	<i>Amplimerlinius macrurus</i>	Germany	Sayre and Starr, 1988
11.	<i>Amplimerlinius</i> sp.	Germany	Sayre and Starr, 1988
12.	<i>Anaplectus grandepapillatus</i>	U.S.A.	Sturhan, 1988
		Germany	Sayre and Starr, 1988
13.	<i>Anaplectus granulosus</i>	Germany	Sayre and Starr, 1988
		Iceland	Sayre and Starr, 1988
14.	<i>Aphasmatylenchus nigeriensis</i>	Liberia	Ciancio et al., 1994
15.	<i>Aphanolaimus</i> sp.	Germany	Sayre and Starr, 1988
16.	<i>Aphelenchoides bicaudatus</i>	Iran	Sayre and Starr, 1988
17.	<i>Aphelenchoides composticola</i>	Germany	Sturhan, 1988
		Iran	Sayre and Starr, 1988
18.	<i>Aphelenchoides dactylocercus</i>	Italy	Roccuzzo and Ciancio, 1991
19.	<i>Aphelenchoides megadorus</i>	U.S.A.	Allen, 1941
20.	<i>Aphelenchoides parietinus</i>	Germany	Steiner, 1938
21.	<i>Aphelenchoides rutgersi</i>	Italy	Ciancio et al., 1994
22.	<i>Aphelenchoides saprophilus</i>	Germany	Sturhan 1988
23.	<i>Aphelenchoides</i> sp.	Germany	Sayre and Starr, 1988
		U.S.A., Florida	Pers. obs.
24.	<i>Aphelenchus avenae</i>	Germany	Sayre and Starr, 1988
25.	<i>Aphelenchus</i> sp.	Mozambique	Sturhan, 1988
26.	<i>Aporcelaimellus</i> cf. <i>simplex</i>	Germany	Sturhan, 1988
27.	<i>Aporcelaimellus obtusicaudatus</i>	Germany	Sturhan, 1988
28.	<i>Aporcelaimus eurydorys</i>	Germany	Sturhan, 1988
		U.S.A., South Dakota	Sayre and Starr, 1988
29.	<i>Aulolaimus bathybius</i>	Germany	Sturhan, 1988
30.	<i>Aulolaimus nannocephalus</i>	Germany	Sturhan, 1988
31.	<i>Aulolaimus oxycephalus</i>	Germany	Sturhan, 1988
32.	<i>Aulolaimus</i> sp.	Germany	Sayre and Starr, 1988
33.	<i>Axonchium nairi</i>	Germany	Sturhan, 1988
34.	<i>Axonchium valvulatum</i>	Sri Lanka	Ciancio et al., 1994
35.	<i>Basiria gracilis</i>	Germany	Sturhan, 1988
36.	<i>Basiria</i> sp.	Finland	Sayre and Starr, 1988
		Germany	Sayre and Starr, 1988
37.	<i>Basirotyleptus penetrans</i>	Nicaragua	Sturhan, 1988
38.	<i>Basirotyleptus</i> sp.	Nicaragua	Sayre and Starr, 1988
39.	<i>Bastiania longicaudata</i>	Germany	Sturhan, 1988

Table 1.2. Continued

No.	Nematode	Location	Source
40.	<i>Belondirella</i> sp.	Nicaragua	Sturhan, 1988
41.	<i>Belonolaimus gracilis</i>	U.S.A., Florida	Sayre and Starr, 1988
42.	<i>Belonolaimus longicaudatus</i>	U.S.A., Florida	Sayre and Starr, 1988
43.	<i>Belonolaimus</i> spp.	U.S.A., Florida	Hewlett et al., 1994
44.	<i>Boleodorus thylactus</i>	France	Sturhan, 1988
		Italy	Ciancio et al., 1994
45.	<i>Cactodera cacti</i> (syn. <i>Heterodera cacti</i>)	Bolivia	Sturhan, 1988
46.	<i>Cephalobus persegnis</i>	Germany	Sayre and Starr, 1988
47.	<i>Cephalobus</i> sp.	U.S.A., Florida	Pers. obs.
48.	<i>Clarkus papillatus</i>	Germany	Sturhan, 1988
		Germany	Sayre and Starr, 1988
49.	<i>Coslenchus andrassyi</i>	Germany	Sturhan, 1988
50.	<i>Coslenchus costatus</i>	Germany	Sayre and Starr, 1988
		Italy	Ciancio et al., 1994
51.	<i>Coslenchus multigyrus</i>	Germany	Sturhan, 1988
52.	<i>Coslenchus turkeyensis</i>	Italy	Ciancio et al., 1994
53.	<i>Criconemella onoensis</i>	Nicaragua	Sayre and Starr, 1988
54.	<i>Criconemella</i> spp.	U.S.A., Florida	Hewlett et al., 1994
	<i>Cylindrolaimus communis</i>	Germany	Sturhan, 1988)
		Italy	Ciancio et al., 1994
55.	<i>Diphtherophora</i> sp.	Germany	Sayre and Starr, 1988
		Iran	Sayre and Starr, 1988
		Germany	Sturhan, 1988
56.	<i>Discocriconemella mauritiensis</i>	South Africa	Sayre and Starr, 1988
		Mauritius	Ciancio et al., 1994
57.	<i>Discolaimium bulbiferum</i>	Iran	Sturhan, 1988
		U.S.A., Hawaii	Sayre and Starr, 1988
58.	<i>Discolaimus carteri</i>	Denmark	Williams, 1960
59.	<i>Discolaimus major</i>	Italy	Ciancio et al., 1994
60.	<i>Discolaimus</i> sp.	Zaire	Sayre and Starr, 1988
61.	<i>Ditylenchus</i> sp.	Germany	Sayre and Starr, 1988
62.	<i>Dolichodorus obtusus</i>	U.S.A., California	Sayre and Starr, 1988
		U.S.A., Florida	Sayre and Starr, 1988
63.	<i>Dolichodorus</i> sp.	Mozambique	Sayre and Starr, 1988
64.	<i>Dorylaimellus demani</i>	Germany	Sturhan, 1988
65.	<i>Dorylaimellus</i> sp.	Chile	Sturhan, 1988
66.	<i>Dorylaimellus virginianus</i>	Switzerland	Sayre and Starr, 1988
67.	<i>Dorylaimida</i>	Azores	Sayre and Starr, 1988
		Germany	Sayre and Starr, 1988
		Iran	Sayre and Starr, 1988
		Madeira Islands	Sayre and Starr, 1988
		Nicaragua	Sayre and Starr, 1988
68.	<i>Dorylaimoides mitis</i>	Ethiopia	Ciancio et al., 1994
69.	<i>Dorylaimus carteri</i>	Denmark	Sayre and Starr, 1988
70.	<i>Dorylaimus</i> sp.	Switzerland	Sayre and Starr, 1988
71.	<i>Doryllium minor</i>	Germany	Sturhan, 1988
72.	<i>Doryllium</i> sp.	Nicaragua	Sturhan, 1988
73.	<i>Ecumenicus monohystera</i> (syn. <i>Eudorylaimus monohystera</i>)	U.S.S.R.	Sturhan, 1988

Table 1.2. Continued

No.	Nematode	Location	Source
75.	<i>Encholaimus taurus</i>	Nicaragua	Sturhan, 1988
76.	<i>Epidorylaimus</i> cf. <i>consobrinus</i>	England	Sturhan, 1988
77.	<i>Eucephalobus oxyuroides</i>	Germany	Sturhan, 1988
78.	<i>Eucephalobus</i> sp.	U.S.A., California	Ciancio and Mankau, 1989b
79.	<i>Eucephalobus striatus</i>	Germany	Sayre and Starr, 1988
80.	<i>Eudorylaimus morbidus</i>	Venezuela	Sayre and Starr, 1988
81.	<i>Eudorylaimus parvus</i>	Germany	Sayre and Starr, 1988
82.	<i>Eudorylaimus</i> sp.	Scotland	Sayre and Starr, 1988
		Brazil	Sturhan, 1988
		France	Sturhan, 1988
		Nicaragua	Sturhan, 1988
83.	<i>Eumonhystera vulgaris</i>	Germany	Sturhan, 1988
84.	<i>Filenchus attenuatus</i>	France	Sturhan, 1988
85.	<i>Filenchus helenae</i>	Germany	Sturhan, 1988
86.	<i>Filenchus</i> sp.	Germany	Sturhan, 1988
		Austria	Sturhan, 1988
		Switzerland	Sturhan, 1988
87.	<i>Filenchus thornei</i>	Germany	Sturhan, 1988
88.	<i>Filenchus vulgaris</i>	Germany	Sturhan, 1988
89.	<i>Funaria maryanneae</i>	Germany	Sturhan, 1988
90.	<i>Geocenamus tenuidens</i>	Germany	Sayre and Starr, 1988
91.	<i>Globodera pallida</i>	England	Davies et al., 1990
		India	Sharma and Davies, 1996
92.	<i>Globodera rostochiensis</i>	Japan	Sayre and Starr, 1988
		England	Davies et al., 1990
		India	Sharma and Davies, 1996
93.	<i>Gylenchulus</i> spp.	U.S.A., Florida	Hewlett et al., 1994
94.	<i>Helicotylenchus californicus</i>	Peru	Ciancio et al., 1994
95.	<i>Helicotylenchus canadensis</i>	Germany	Sayre and Starr, 1988
96.	<i>Helicotylenchus</i> cf. <i>microcephalus</i>	Mozambique	Sturhan, 1988
97.	<i>Helicotylenchus crenacauda</i>	Algeria	Ciancio et al., 1994
98.	<i>Helicotylenchus digonicus</i>	Germany	Sayre and Starr, 1988
		Hungary	Ciancio et al., 1994
		Malta	Ciancio et al., 1994
		Croatia	Ciancio et al., 1994
		Switzerland	Sayre and Starr, 1988
		Italy	Ciancio et al., 1994
		Algeria	Ciancio et al., 1994
99.	<i>Helicotylenchus dihystra</i>	Azores	Sayre and Starr, 1988
		Algeria	Ciancio et al., 1994
		South Africa	Sayre and Starr, 1988
		U.S.A., Florida	Sayre and Starr, 1988
100.	<i>Helicotylenchus erythrinae</i>	Madeira Islands	Sayre and Starr, 1988
101.	<i>Helicotylenchus krugeri</i>	South Africa	Sayre and Starr, 1988
102.	<i>Helicotylenchus lobus</i>	U.S.A., California	Ciancio et al., 1992)
103.	<i>Helicotylenchus microlobus</i>	U.S.A., Florida	Sayre and Starr, 1988
104.	<i>Helicotylenchus paxilli</i>	Germany	Sayre and Starr, 1988
105.	<i>Helicotylenchus pseudodigonicus</i>	Germany	Sayre and Starr, 1988

Table 1.2. Continued

No.	Nematode	Location	Source
106.	<i>Helicotylenchus pseudorobustus</i>	Azores Algeria Germany Greece Iran Italy Madeira Islands Peru	Sayre and Starr, 1988 Ciancio et al., 1994 Sayre and Starr, 1988 Volvass et al., 1993 Sayre and Starr, 1988 Ciancio et al., 1994 Sayre and Starr, 1988 Ciancio et al., 1994
107.	<i>Helicotylenchus</i> sp.	Azores Brazil Canary Islands Dominican Republic Germany Haiti India Ivory Coast U.S.A., Florida	Sayre and Starr, 1988 Sayre and Starr, 1988 Sayre and Starr, 1988 Sayre and Starr, 1988 Sayre and Starr, 1988 Sayre and Starr, 1988 Sayre and Starr, 1988 Sturhan, 1988 Hewlett et al., 1994
108.	<i>Helicotylenchus variocaudatus</i> (syn. <i>Rotylenchoides variocaudatus</i>)	Germany Ivory Coast	Sayre and Starr, 1988 Sturhan, 1988
109.	<i>Helicotylenchus vulgaris</i>	Germany Algeria Italy Romania	Sayre and Starr, 1988 Ciancio et al., 1994 Ciancio et al., 1994 Sayre and Starr, 1988
110.	<i>Hemicycliophora</i> spp.	U.S.A., Florida	Hewlett et al., 1994
111.	<i>Heterodera avenae</i>	Germany England	Sayre and Starr, 1988 Davies et al., 1990
112.	<i>Heterodera cacti</i>	Bolivia	Ciancio and Mankau, 1989a
113.	<i>Heterodera cajani</i>	India	Sharma and Sharma, 1989
114.	<i>Heterodera elachista</i>	Japan	Sayre and Starr, 1988
115.	<i>Heterodera fici</i>	Italy	De-O-Abrantes and Vovlas, 1988
116.	<i>Heterodera glycines</i>	Japan India England U.S.A., Illinois	Sayre and Starr, 1988 Sharma and Davies, 1996 Davies et al., 1990 Noel and Stanger, 1994
117.	<i>Heterodera goettingiana</i>	Germany	Sayre and Starr, 1988
118.	<i>Heterodera leuceilyma</i>	U.S.A., Florida	Sayre and Starr, 1988
119.	<i>Heterodera schachtii</i>	Germany India	Sturhan, 1988 Sharma and Davies, 1996
120.	<i>Heterodera</i> sp.	Germany Nicaragua India U.S.A., Florida	Sayre and Starr, 1988 Sayre and Starr, 1988 Sturhan, 1988 Hewlett et al., 1994
121.	<i>Heterodera trifolii</i>	India	Sharma and Davies, 1996
122.	<i>Hirschmanniella gracilis</i>	Germany U.S.A., Florida	Sayre and Starr, 1988 Sayre and Starr, 1988
123.	<i>Hirschmanniella mucronata</i>	Philippines	Sturhan, 1988
124.	<i>Hirschmanniella oryzae</i>	Philippines	Sturhan, 1988
125.	<i>Histotylenchus histoides</i>	South Africa	Sayre and Starr, 1988
126.	<i>Hoplotaimus galeatus</i>	U.S.A., Florida	Sayre and Starr, 1988

Table 1.2. Continued

No.	Nematode	Location	Source
127.	<i>Hoplotaimus indicus</i>	India	Sayre and Starr, 1988
128.	<i>Hoplotaimus</i> sp.	U.S.A., California	Sayre and Starr, 1988
		U.S.A., Florida	Sayre and Starr, 1988
129.	<i>Hoplotaimus</i> spp.	U.S.A., Florida	Hewlett et al., 1994
130.	<i>Hoplotaimus tylenchiformis</i> (= <i>Rotylenchus robustus</i>)	U.S.A., Florida	Sayre and Starr, 1988
131.	<i>Hoplotaimus uniformis</i>	Netherlands	Sayre and Starr, 1988
		Iran	Sayre and Starr, 1988
		Mozambique	Sayre and Starr, 1988
		Nigeria	Sayre and Starr, 1988
		Samoa	Sayre and Starr, 1988
		U.S.A.	Sayre and Starr, 1988
132.	<i>Hoplotylus montanus</i>	Japan	Sturhan, 1988
133.	<i>Hoplotylus silvaticus</i>	U.S.A.	Sturhan, 1988
134.	<i>Hypsoperine</i> spp.	U.S.A., Florida	Hewlett et al., 1994
135.	<i>Ironus ignavus</i>	Sweden	Sayre and Starr, 1988
136.	<i>Isolaimium nigeriense</i>	Nigeria	Sayre and Starr, 1988
137.	<i>Laimydorus reversus</i>	U.S.A., South Dakota	Sayre and Starr, 1988
138.	<i>Limonchulus bryophilus</i>	Nicaragua	Sturhan, 1988
139.	<i>Longidorella europaea</i>	Germany	Sturhan, 1988
140.	<i>Longidorella morbidus</i>	Venezuela	Ciancio and Mankau, 1989a
141.	<i>Longidorella parva</i>	Italy	Ciancio et al., 1994
142.	<i>Longidorella</i> sp.	Germany	Sayre and Starr, 1988
		Italy	Ciancio et al., 1994
143.	<i>Longidorus attenuatus</i>	Romania	Ciancio et al., 1994
144.	<i>Longidorus caespiticola</i>	Germany	Sayre and Starr, 1988
145.	<i>Longidorus elongatus</i>	Germany	Sayre and Starr, 1988
146.	<i>Longidorus euonymus</i>	Bulgaria	Sturhan, 1988
		Italy	Ciancio et al., 1994
147.	<i>Longidorus laevicapitatus</i>	Liberia	Ciancio et al., 1994
		Ethiopia	Ciancio et al., 1994
148.	<i>Longidorus leptocephalus</i>	Germany	Sayre and Starr, 1988
149.	<i>Longidorus profundorum</i>	Germany	Sayre and Starr, 1988
150.	<i>Longidorus</i> sp.	Sri Lanka	Ciancio et al., 1994
151.	<i>Longidorus vineacola</i>	Germany	Sayre and Starr, 1988
152.	<i>Megadorus megadorus</i>	U.S.A., Utah	Sayre and Starr, 1988
153.	<i>Meloidodera floridensis</i>	U.S.A., Florida	Sayre and Starr, 1988
154.	<i>Meloidodera</i> spp.	U.S.A., Florida	Hewlett et al., 1994
155.	<i>Meloidoderita</i> sp.	Iran	Sayre and Starr, 1988
156.	<i>Meloidogyne acrita</i>	U.S.A., Florida	Sayre and Starr, 1988
157.	<i>Meloidogyne acronoea</i>	Malawi	Sturhan, 1988
158.	<i>Meloidogyne ardenensis</i>	Germany	Sayre and Starr, 1988
159.	<i>Meloidogyne arenaria</i>	Netherlands	Sayre and Starr, 1988
		China	Pan et al., 1993
		Spain	Verdejo-Lucas, 1992
		U.S.A., California	Sayre and Starr, 1988
		U.S.A., Florida	Sayre and Starr, 1988
160.	<i>Meloidogyne coffeicola</i>	Brazil	Sayre and Starr, 1988
161.	<i>Meloidogyne exigua</i>	Colombia	Sayre and Starr, 1988

Table 1.2. Continued

No.	Nematode	Location	Source
162.	<i>Meloidogyne fujianensis</i>	China	Pan et al., 1993
163.	<i>Meloidogyne graminis</i>	Germany	Sayre and Starr, 1988
164.	<i>Meloidogyne hapla</i>	Japan	Sayre and Starr, 1988
		China	Pan et al., 1993
		New Zealand	Watson et al., 1990
		Spain	Verdejo-Lucas, 1992
		U.S.A., California	Sayre and Starr, 1988
		U.S.A., Maryland	Sayre and Starr, 1988
165.	<i>Meloidogyne incognita</i>	India	Bhattacharya and Swarup, 1988
		China	Pan et al., 1993
		Colombia	Ciancio and Mankau, 1989a
		Japan	Sayre and Starr, 1988
		Mauritius	Sayre and Starr, 1988
		Pakistan	Maqbool and Zaki, 1990
		Puerto Rico	Vargas et al., 1992
		Spain	Verdejo-Lucas, 1992
		South Africa	Sayre and Starr, 1988
		Togo	Sayre and Starr, 1988
		Germany	Sturhan, 1988
		U.S.A., California	Sayre and Starr, 1988
		U.S.A., Florida	Sayre and Starr, 1988
		U.S.A., Louisiana	Sayre and Starr, 1988
		U.S.A., Maryland	Sayre and Starr, 1988
166.	<i>Meloidogyne javanica</i>	Australia	Sayre and Starr, 1988
		Brazil	Sayre and Starr, 1988
		China	Pan et al., 1993
		India	Sayre and Starr, 1988
		Japan	Sayre and Starr, 1988
		Mauritius	Sayre and Starr, 1988
		Pakistan	Maqbool and Zaki, 1990
		U.S.A., California	Sayre and Starr, 1988
		U.S.A., Florida	Sayre and Starr, 1988
		U.S.A., Maryland	Sayre and Starr, 1988
167.	<i>Meloidogyne lusitanica</i>	Portugal	Abrantes and Vovlas, 1988
168.	<i>Meloidogyne naasi</i>	Finland	Sayre and Starr, 1988
		Germany	Sayre and Starr, 1988
169.	<i>Meloidogyne</i> sp.	Germany	Sayre and Starr, 1988
		Nicaragua	Sayre and Starr, 1988
		Cuba	Sturhan, 1988
		Portugal	Oostendorp et al., 1990
170.	<i>Meloidogyne</i> spp.	Puerto Rico	Vargas and Acosta, 1990
		China	Lin and Chen, 1992
171.	<i>Merlinius bavaricus</i>	Germany	Sayre and Starr, 1988
172.	<i>Merlinius brevidens</i>	Germany	Sayre and Starr, 1988
		Italy	Sayre and Starr, 1988
		Madeira Islands	Sayre and Starr, 1988
173.	<i>Merlinius joctus</i>	U.S.A.	Sturhan, 1988
		Germany	Sayre and Starr, 1988

Table 1.2. Continued

No.	Nematode	Location	Source
174.	<i>Merlinius macrurus</i> (= <i>Amplimerlinius macrurus</i>)	U.S.A., Florida	Sayre and Starr, 1988
175.	<i>Merlinius microdorus</i>	Germany	Sayre and Starr, 1988
		Iran	Sayre and Starr, 1988
176.	<i>Merlinius nanus</i>	Germany	Sayre and Starr, 1988
177.	<i>Merlinius nothus</i>	Germany	Sayre and Starr, 1988
178.	<i>Merlinius processus</i>	Germany	Sayre and Starr, 1988
179.	<i>Merlinius</i> sp.	Germany	Sayre and Starr, 1988
		Iran	Sayre and Starr, 1988
		Italy	Ciancio et al., 1994
180.	<i>Merlinius stegus</i>	Iran	Barooti, 1989
181.	<i>Merlinius tessellatus</i> (= <i>Scutylenchus tessellatus</i>)	Netherlands	Sayre and Starr, 1988
182.	<i>Mesocriconema ornatum</i>	U.S.A., Florida	Dickson (pers. comm.)
183.	<i>Mesodorylaimus</i> cf. <i>bastiani</i>	Germany	Sturhan, 1988
184.	<i>Mesorhabditis</i> sp.	Germany	Sayre and Starr, 1988
185.	<i>Monhystera paludicola</i>	Denmark	Williams, 1960
186.	<i>Mononchus papillatus</i> (= <i>Clarkus papillatus</i>)	Scotland	Sayre and Starr, 1988
187.	<i>Mumtazium mumtazae</i>	Uganda	Sayre and Starr, 1988
188.	<i>Mylonchulus boveyi</i>	U.S.A.	Ciancio et al., 1994
189.	<i>Mylonchulus brachyuris</i>	Germany	Sayre and Starr, 1988
190.	<i>Nagelus camelliae</i>	Iran	Sayre and Starr, 1988
191.	<i>Nagelus leptus</i>	Germany	Sayre and Starr, 1988
		Iceland	Sayre and Starr, 1988
192.	<i>Neopsilenchus magnidens</i>	France	Sturhan, 1988
193.	<i>Nygolaimus parabrachyurus</i>	U.S.A., South Dakota	Sayre and Starr, 1988
194.	<i>Nygolaimus</i> sp.	Germany	Sayre and Starr, 1988
195.	<i>Opisthodorylaimus sylphoides</i>	Italy	Ciancio et al., 1994
196.	<i>Oxydirus oxycephalus</i>	Germany	Sayre and Starr, 1988
197.	<i>Paralongidorus citri</i>	Sri Lanka	Ciancio et al., 1994
198.	<i>Paralongidorus sali</i>	India	Sayre and Starr, 1988
		U.S.A., Florida	Sayre and Starr, 1988
199.	<i>Paraphelenchulus pseudoparietinus</i>	Germany	Sayre and Starr, 1988
200.	<i>Paratrichodorus minor</i> (syn. <i>Trichodorus christiei</i>)	U.S.A.	Sturhan, 1988
201.	<i>Paratrichodorus</i> spp.	U.S.A., Florida	Hewlett et al., 1994
202.	<i>Paratrophurus anomalus</i>	Algeria	Ciancio et al., 1994
		São Tomé	Ciancio et al., 1994
203.	<i>Paratylenchus bukowinensis</i>	Germany	Sayre and Starr, 1988
204.	<i>Paratylenchus mutabilis</i> (syn. <i>Gracilacus mutabilis</i>)	Australia	Sturhan, 1988
205.	<i>Paratylenchus pandata</i> (syn. <i>Gracilacus pandata</i>)	Nigeria	Sturhan, 1988
206.	<i>Paratylenchus</i> sp.	Germany	Sayre and Starr, 1988
		Canada	Sturhan, 1988
		Nicaragua	Sturhan, 1988
207.	<i>Paratylenchus straeleni</i>	Germany	Sayre and Starr, 1988
208.	<i>Plectus acuminatus</i>	Germany	Sayre and Starr, 1988
209.	<i>Plectus cirratus</i>	Germany	Sayre and Starr, 1988

Table 1.2. Continued

No.	Nematode	Location	Source
210.	<i>Plectus rhizophilus</i>	Germany	Sayre and Starr, 1988
211.	<i>Plectus</i> sp.	Germany	Sayre and Starr, 1988
212.	<i>Pratylenchoides</i> cf. <i>bacilisemenus</i>	Canada	Sturhan, 1988
213.	<i>Pratylenchoides crenicauda</i>	Germany	Sayre and Starr, 1988
214.	<i>Pratylenchoides laticauda</i>	Germany	Sayre and Starr, 1988
215.	<i>Pratylenchoides</i> sp.	Canada	Sayre and Starr, 1988
		Finland	Sayre and Starr, 1988
		Germany	Sayre and Starr, 1988
		Iran	Sayre and Starr, 1988
		Italy	Sayre and Starr, 1988
216.	<i>Pratylenchus brachyurus</i>	U.S.A., Florida	Sayre and Starr, 1988
		U.S.A., Georgia	Sayre and Starr, 1988
		U.S.A., Maryland	Sayre and Starr, 1988
		U.S.A., South Carolina	Sayre and Starr, 1988
		Ivory Coast	Sturhan, 1988
217.	<i>Pratylenchus convallariae</i>	Germany	Sayre and Starr, 1988
218.	<i>Pratylenchus crenatus</i>	Germany	Sayre and Starr, 1988
219.	<i>Pratylenchus fallax</i>	Germany	Sayre and Starr, 1988
220.	<i>Pratylenchus flakkensis</i>	Germany	Sayre and Starr, 1988
		England	Sturhan, 1988
221.	<i>Pratylenchus neglectus</i>	Austria	Sayre and Starr, 1988
		Croatia	Ciancio et al., 1994
		Germany	Sayre and Starr, 1988
		Italy	Ciancio et al., 1994
222.	<i>Pratylenchus penetrans</i>	Germany	Sayre and Starr, 1988
		Netherlands	Sayre and Starr, 1988
		U.S.A., Florida	Sayre and Starr, 1988
223.	<i>Pratylenchus pratensis</i>	Germany	Sayre and Starr, 1988
		Netherlands	Sayre and Starr, 1988
224.	<i>Pratylenchus scribneri</i>	U.S.A., California	Sayre and Starr, 1988
		U.S.A., Florida	Oostendorp et al., 1990
225.	<i>Pratylenchus</i> sp.	Germany	Sayre and Starr, 1988
		Greece	Sayre and Starr, 1988
		U.S.A., Florida	Sayre and Starr, 1988
		U.S.A., Oregon	Sayre and Starr, 1988
226.	<i>Pratylenchus</i> spp.	U.S.A., Illinois	Sayre and Starr, 1988
		U.S.A., Maryland	Sayre and Starr, 1988
		U.S.A., Florida	Hewlett et al., 1994
227.	<i>Pratylenchus thornei</i>	Germany	Sayre and Starr, 1988
228.	<i>Pratylenchus zeae</i>	Dominican Republic	Sayre and Starr, 1988
		Mozambique	Sayre and Starr, 1988
		South Africa	Sayre and Starr, 1988
		U.S.A., Florida	Sayre and Starr, 1988
229.	<i>Prionchulus</i> sp.	Germany	Sayre and Starr, 1988
230.	<i>Pungentus engadinensis</i>	Germany	Sturhan, 1988
231.	<i>Pungentus silvaticus</i>	Azores	Sturhan, 1988
232.	<i>Pungentus</i> sp.	Germany	Sayre and Starr, 1988
233.	<i>Quinisulcius curvus</i>	Dominican Republic	Sayre and Starr, 1988

Table 1.2. Continued

No.	Nematode	Location	Source
234.	<i>Quinisulcius sulcatus</i>	Israel	Sayre and Starr, 1988
235.	<i>Radopholus gracilis</i> (= <i>Hirschmanniella gracilis</i>)	Germany	Sayre and Starr, 1988
236.	<i>Rhabditis</i> sp.	Germany	Sayre and Starr, 1988
237.	<i>Rotylenchulus reniformis</i>	India	Sharma and Davies, 1996
238.	<i>Rotylenchus capensis</i>	Greece	Volvas et al., 1993
239.	<i>Rotylenchus fallorobustus</i>	Germany	Sayre and Starr, 1988
240.	<i>Rotylenchus goodeyi</i>	Germany	Sayre and Starr, 1988
241.	<i>Rotylenchus laurentinus</i>	Italy	Ciancio et al., 1994
242.	<i>Rotylenchus incultus</i>	South Africa	Sayre and Starr, 1988
243.	<i>Rotylenchus quartus</i>	Germany	Sayre and Starr, 1988
244.	<i>Rotylenchus robustus</i>	Switzerland	Sayre and Starr, 1988
		Netherlands	Sayre and Starr, 1988
		U.S.A., Florida	Sayre and Starr, 1988
245.	<i>Rotylenchus</i> sp.	Germany	Sayre and Starr, 1988
		Israel	Sayre and Starr, 1988
246.	<i>Rotylenchus unisexus</i>	South Africa	Sayre and Starr, 1988
247.	<i>Scutellonema brachyurum</i>	South Africa	Sayre and Starr, 1988
248.	<i>Scutellonema clathrycaudatum</i>	Sierra Leone	Sayre and Starr, 1988
249.	<i>Scutellonema quadrifer</i>	Germany	Sayre and Starr, 1988
250.	<i>Scutellonema rugosus</i>	Iran	Sayre and Starr, 1988
251.	<i>Scutellonema</i> sp.	Nigeria	Sayre and Starr, 1988
		Malawi	Sturhan, 1988
252.	<i>Scutellonema</i> spp.	U.S.A., Florida	Sayre and Starr, 1988
253.	<i>Scutellonema truncatum</i>	South Africa	Sayre and Starr, 1988
254.	<i>Scutylenchus</i> sp.	Germany	Sayre and Starr, 1988
		Iran	Sayre and Starr, 1988
255.	<i>Scutylenchus tessellatus</i>	Germany	Sayre and Starr, 1988
256.	<i>Seimura tenuicaudata</i>	Germany	Sturhan, 1988
257.	<i>Sphaeronema californicum</i>	Canada	Sayre and Starr, 1988
258.	<i>Sphaeronema rumicis</i>	Germany	Sayre and Starr, 1988
259.	<i>Steinernema glaseri</i>	U.S.A., Texas	Knoung, 1996
260.	<i>Tanzanius coffeae</i>	Tanzania	Siddiqi, 1991
261.	<i>Thonus circulifer</i>	Germany	Sturhan, 1988
262.	<i>Trichodorus similis</i>	Germany	Sayre and Starr, 1988
263.	<i>Trichodorus sparsus</i>	Germany	Sayre and Starr, 1988
264.	<i>Trilobus medius</i>	Denmark	Williams, 1960
265.	<i>Tripyla monohystera</i>	U.S.A.,	Nishizawa, 1989
266.	<i>Trophonema okamotoi</i>	U.S.A., Florida	Inserra et al., 1992
267.	<i>Tylencholaimus minimus</i>	Germany	Sayre and Starr, 1988
268.	<i>Tylencholaimus</i> sp.	Azores	Sturhan, 1988
269.	<i>Tylenchorhynchus annulatus</i> (syn. <i>T. martini</i>)	U.S.A., Florida	Giblin-Davis et al, 1990
		U.S.A.	Sturhan, 1988
270.	<i>Tylenchorhynchus brassicae</i>	Canary Islands	Sayre and Starr, 1988

Table 1.2. Continued

No.	Nematode	Location	Source
271.	<i>Tylenchorhynchus dubius</i>	Belgium	Sayre and Starr, 1988
		Germany	Sayre and Starr, 1988
		Netherlands	Sayre and Starr, 1988
		Scotland	Sayre and Starr, 1988
		U.S.A., Florida	Sayre and Starr, 1988
272.	<i>Tylenchorhynchus lamelliferus</i>	Germany	Sayre and Starr, 1988
273.	<i>Tylenchorhynchus maximus</i>	Germany	Sayre and Starr, 1988
		U.S.A., Maryland	Sayre and Starr, 1988
274.	<i>Tylenchorhynchus maximus</i>	Austria	Sturhan, 1988
275.	<i>Tylenchorhynchus microphasmis</i>	Germany	Sayre and Starr, 1988
276.	<i>Tylenchorhynchus nanus</i> (= <i>Merlinius nanus</i>)	Belgium	Sayre and Starr, 1988
		U.S.A., Florida	Sayre and Starr, 1988
277.	<i>Tylenchorhynchus nudus</i>	U.S.A., South Dakota	Sayre and Starr, 1988
278.	<i>Tylenchorhynchus</i> sp.	Cuba	Sturhan, 1988
		U.S.A., Colorado	Sayre and Starr, 1988
279.	<i>Tylenchorhynchus</i> spp.	U.S.A., Florida	Hewlett et al., 1994
280.	<i>Tylenchulus semipenetrans</i>	Samoa	Sayre and Starr, 1988
		France	Sturhan, 1988
		Iraq	Fattah et al., 1989
		Iran	Maafi, 1993
		Italy	Ciancio et al., 1994
		U.S.A., Florida	Kaplan, 1994
281.	<i>Tylenchulus</i> sp.	South Africa	Sayre and Starr, 1988
		Finland	Sayre and Starr, 1988
		Iceland	Sayre and Starr, 1988
		Netherlands	Sayre and Starr, 1988
		Romania	Sayre and Starr, 1988
		U.S.A., Florida	Sayre and Starr, 1988
282.	<i>Tylenchus elegans</i>	Iceland	Sturhan, 1988
283.	<i>Tylenchus</i> sp.	Iran	Sturhan, 1988
284.	<i>Tylenchus</i> spp.	Azores	Sayre and Starr, 1988
		Germany	Sayre and Starr, 1988
285.	<i>Tyolaimophorus minor</i>	Germany	Sturhan, 1988
286.	<i>Xiphinema americanum</i>	Sri Lanka	Sayre and Starr, 1988
		U.S.A.	Sayre and Starr, 1988
		Peru	Ciancio and Mankau, 1989a
287.	<i>Xiphinema bakeri</i>	Canada	Sayre and Starr, 1988
		Peru	Ciancio and Mankau, 1989a
288.	<i>Xiphinema basiri</i>	Liberia	Ciancio et al., 1994
289.	<i>Xiphinema bergeri</i>	South Korea	Ciancio et al., 1994
290.	<i>Xiphinema brasiliense</i>	Peru	Ciancio and Mankau, 1989a
291.	<i>Xiphinema brevicolle</i>	Poland	Ciancio et al., 1994
292.	<i>Xiphinema</i> cf. <i>imitator</i>	South Africa	Sayre and Starr, 1988
293.	<i>Xiphinema chambersi</i>	U.S.A., South Dakota	Sayre and Starr, 1988
		Peru	Ciancio and Mankau, 1989a
294.	<i>Xiphinema coxi</i>	Germany	Sayre and Starr, 1988
		Peru	Ciancio and Mankau, 1989a

Table 1.2. Continued

No.	Nematode	Location	Source
294.	<i>Xiphinema diversicaudatum</i>	Germany	Sayre and Starr, 1988
		Italy	Ciancio, 1995
		Peru	Ciancio and Mankau, 1989a
295.	<i>Xiphinema ebriense</i>	Liberia	Ciancio et al., 1994
296.	<i>Xiphinema elongatum</i>	Mauritius	Sayre and Starr, 1988
		Philippines	Ciancio et al., 1994
		South Africa	Sayre and Starr, 1988
		Sri Lanka	Ciancio et al., 1994
		U.S.A., Florida	Sayre and Starr, 1988
297.	<i>Xiphinema ifacolum</i>	Liberia	Ciancio et al., 1994
298.	<i>Xiphinema imitator</i>	South Africa	Ciancio and Mankau, 1989a
299.	<i>Xiphinema index</i>	Iran	Sayre and Starr, 1988
300.	<i>Xiphinema ingens</i>	Ethiopia	Ciancio et al., 1994
301.	<i>Xiphinema insigne</i>	Ethiopia	Ciancio et al., 1994
302.	<i>Xiphinema longicaudatum</i>	Liberia	Ciancio et al., 1994
303.	<i>Xiphinema magaliesmontanum</i>	South Africa	Sturhan, 1988
304.	<i>Xiphinema pachtaicum</i>	Canary Islands	Sturhan, 1988
		Iran	Sayre and Starr, 1988
		Hungary	Ciancio et al., 1994
		Italy	Ciancio et al., 1994
305.	<i>Xiphinema pseudocoxi</i>	Germany	Sayre and Starr, 1988
306.	<i>Xiphinema radiculicola</i>	Sri Lanka	Ciancio et al., 1994
307.	<i>Xiphinema rivesi</i>	U.S.A.	Sturhan, 1988
308.	<i>Xiphinema rotundatum</i>	Liberia	Ciancio et al., 1994
309.	<i>Xiphinema setariae</i>	Ethiopia	Ciancio et al., 1994
		Sri Lanka	Ciancio et al., 1994
310.	<i>Xiphinema</i> sp.	Azores	Sayre and Starr, 1988
		Zaire	Sayre and Starr, 1988
		Liberia	Ciancio et al., 1994
		Somalia	Ciancio et al., 1994
		U.S.A., Florida	Hewlett et al., 1994
311.	<i>Xiphinema turcicum</i>	Israel	Ciancio et al., 1994
312.	<i>Xiphinema vuittenezi</i>	Hungary	Ciancio et al., 1994
313.	<i>Zygotylenchus guevarai</i>	Germany	Sturhan, 1988

list includes 14 new genera, 57 new species, 14 new countries, and 138 new geographical distribution records.

Host Specificity

Since the biocontrol potential of *Pasteuria* sp. is dependent to some extent on the range of nematodes that it can attack, its host specificity has received considerable attention. However, much of the published data should be treated with caution. Host specificity generally has been obtained by observing attachment of endospores to the cuticles of nematodes rather than by establishing parasitism by actual infection and development inside the nematodes. Genetically diverse endospore populations are commonly used in host range tests; no one has reported work with single endospore isolates of *Pasteuria* sp. Data on endospore attachment typically have been subjected to analysis of variances without a pre-transformation, which gives rise to biased results because the variances associated with the mean endospore attachment have a binomial distribution (Chapter 7). Therefore, there is considerable variation, notably among different host range tests, among duplicated tests, among different isolates of *Pasteuria* sp., and among different generations of the same host nematode line. Host specificity of a particular isolate of *Pasteuria* sp. can be 'trained' and shifted from one nematode host to another by continually culturing the bacterium on the nematode (Davies et al., 1988). After culturing treatments, endospores attached more to the nematode from which they were originally isolated or cultured than to others. In contrast, tests with isolates from *M. javanica* and *M. incognita* by Stirling (1985) showed that endospore attachment is not always related to the species from which the endospores are obtained, or to the species of the recipient nematode.

Sayre and Starr (1985; 1989) concluded that the host range of *P. penetrans* isolates is limited to *Meloidogyne* spp. Stirling (1985) speculated that host specificity of *P. penetrans* might be a response to nematode populations rather than to nematode species. A recent study

shows that *P. penetrans* can produce heterogeneous endospores (Davies et al., 1994). These heterogeneous subpopulations of endospores show specificity to various nematode populations. Therefore, *P. penetrans* may have infinite genomic types that evolve continually to result in endospores capable of attaching to and infecting the nematodes that are present in a particular environment.

The true nature of host specificity in *P. penetrans* still remains to be elucidated. Recent results suggest that proteins on the endospore surface may be involved (Davies et al., 1992; Davies, 1994). Therefore, different endospore attachments among isolates of *P. penetrans* may be due to the quantitative and qualitative differences in these proteins.

Cultivation

Current methods of mass producing *P. penetrans* rely on the multiplication of the pathogen in its nematode host on greenhouse-grown plants (Stirling and Wachtel, 1980). Such production systems might be improved by culturing the nematode and pathogen in excised or transformed root cultures (Verdejo and Jaffee, 1988; Verdejo and Mankau, 1986), but commercial use of the pathogen will most likely require an *in vitro* method of cultivation.

Verdejo and Mankau (1986) developed a method to grow *P. penetrans* in *M. incognita* on excised tomato roots. A *P. penetrans* infected female was squashed on a small block of agar placed close to the roots to release the endospores, then a single *M. incognita* egg mass was placed on the agar block. Endospores attached to J2, which in turn invaded the roots. Diseased females were found after 58 days. The culture was improved using a four-element system containing *M. incognita*, *P. penetrans*, and tomato roots transformed with *Agrobacterium rhizogenes* (Verdejo and Jaffee, 1988).

Reise et al. (1988) tested various media for their ability to support the growth of *Pasteuria* spp. isolates. *Pasteuria* spp. isolates from *Pratylenchus brachyurus*, *Heterodera*

glycines, and *Meloidogyne incognita* were studied. The standard medium formulations were modified by adding undefined organic, defined organic and mineral supplements, which resulted in improved growth. Diseased nematodes were surface sterilized and then crushed in the various media. Increased production of mature endospores, sporangia, and vegetative cells was observed. Growth, closely resembling bacterial structures found in diseased nematodes, gradually decreased to marginal growth, ceasing after three to five transfers.

Williams et al. (1989) tried to culture *P. penetrans* outside of its nematode host, but were not successful, despite using a wide range of simple and complex media. These included media developed to cultivate fastidious organisms such as *Spiroplasma citri*, *Legionella pneumophila*, and *Clavibacter xyli* subsp. *xyli*; media containing root extract, soil extract or crushed nematodes; media suitable for the growth of nematodes such as *Caenorhabditis briggsae*; and media containing compounds such as sterols, which are essential for nematode growth. Either endospores or vegetative mycelial bodies was used as the initial inoculum.

Bishop and Ellar (1991) screened several microbial media for their ability to maintain *in vitro* vegetative forms of *P. penetrans*. No replication of the bacterium was ever observed in these media and lysis of the inoculated bacteria occurred rapidly. Two defined media were then formulated; medium 1 maintained inoculated “ball-mycelia” of *P. penetrans* in an apparently viable state for up to 1 month with low yields of mature endospores and medium 2 gave a small increase in the number of inoculated “ball mycelia” but lysis resulted. Similar results were obtained with cell-free extracts of a fungus and *Thermoactinomyces vulgaris*. Cell-free extracts from other organisms were screened, but without success.

Ecology of *Pasteuria penetrans*

Temperature

The development of *P. penetrans* in nematode hosts was affected by temperature. The minimal developmental temperature was recently determined as 17 °C (Chen and Dickson, unpubl.). Increasing temperature accelerated the development rate of *P. penetrans* (Hatz and Dickson, 1992; Serracin-Ulate, 1995; Serracin et al., 1997). The bacterium developed more quickly within its host at 30 and 35 °C than at 25 °C or below. Mature endospores were observed after 35, 40, 81, and 116 days at 35, 30, 25, and 20 °C, respectively (Hatz and Dickson, 1992), and 28, 35, and 90 days at 35, 28, and 21 °C, respectively, in another experiment (Serracin-Ulate, 1995; Serracin et al., 1997). *Pasteuria penetrans* is a mesophilic bacterium, with optimal growth temperature at 35 °C (Hatz and Dickson, 1992). Development of *P. penetrans* within females of *M. javanica* and *M. arenaria* was not observed at 10 °C (Hatz and Dickson, 1992). Different temperature requirements, however, exist for various isolates of the bacterium because of its cosmopolitan distribution. As an example, an Indian isolate of *P. penetrans* that infects both *Heterodera* spp. and *M. incognita* completed its life cycle in *M. incognita* in 49 days at 10 to 17 °C (Bhattacharya and Swarup, 1988).

Endospore attachment to J2 also was affected by temperature (Ahmed et al., 1990; Singh and Dhawan, 1990; Stirling et al., 1990). Stirling et al. (1990) observed that the rate of endospore attachment at 27 °C was approximately double that at 18 °C. Endospore attachment of J2 increased with increasing temperature. The maximum number of *P. penetrans* endospores attaching to J2 of *M. javanica* was observed at 30 °C (Ahmed et al., 1990; Hatz and Dickson, 1992). Above 30 °C, the number of endospores attached per J2 declined (Hatz and Dickson, 1992). An isolate of *Pasteuria* sp. parasitic on *H. cajani* showed higher numbers of endospores

attached to *H. cajani* at 25 °C than at 15 or 35 °C (Singh and Dhawan, 1990).

Germination of endospores was favored by high temperature. Germ tubes were formed and they penetrated the cuticle and hypodermis of J2 of *M. arenaria* race 2 approximately 9 to 10, 6, and 4 to 5 days after inoculation, at 21, 28, and 35 °C, respectively (Serracin-Ulate, 1995; Serracin et al., 1997). Germination of endospores on *M. incognita* occurred 6 to 8 days after inoculation at 25 °C (Sayre and Wergin, 1977). The mechanism by which temperature causes these effects is unclear; however, temperature may affect the development of host nematodes, which, in turn, triggers the germination of endospores.

Pathogenesis also was favored by high temperature. At 30 °C, *P. penetrans* proliferated extensively through females before they reached maturity, whereas at 20 °C, females often developed ovaries containing eggs before infection prevented further development (Stirling, 1981).

Numbers of endospores per root system also were related to temperature. At temperatures of 20, 25, 30, and 35 °C, the average number of endospores per root system was 12.5, 14.7, 115, and 113 million, respectively (Hatz and Dickson, 1992). The number of endospores per root system also was increased with increasing degree-days (Chapter 5).

Moisture

Little is known about the effect of soil moisture on endospore attachment and development of *P. penetrans*. Endospores of *P. penetrans* are not motile in soil, and endospore attachment to J2 was correlated to the movement of J2 in soil. Because the movement of nematodes through soil is affected by soil moisture conditions (Van Gundy, 1985), endospore attachment should also be affected. Experimentally, however, such correlations were not observed between the number of endospores attached per J2 and the soil pore size or soil moisture levels (Dutky and Sayre, 1978). An experiment to determine whether moistening air-

dried soil containing *P. penetrans* for 3 days before adding J2 of *M. incognita* increased endospore attachment showed that in each case the proportion of J2 with attached endospores was greater in moistened soil (Brown and Smart, 1984). Interestingly, however, soil moisture affects the growth of *P. penetrans* within *Meloidogyne* sp. females. The rate of development of *P. penetrans* in infected females was reduced when soils were maintained at or near field capacity (Davies et al., 1991). Although the reasons for this effect are not known, it is possible that oxygen depletion in wet soil inhibits respiration, resulting in an inhibition of development of both the nematode and the bacterial parasite.

pH

Endospore attachment was affected by pH. The highest attachment rate was at pH 9 (Ahmed et al., 1990) and decreased at low pH values (Ratnasoma, 1990). Davies et al. (1988), however, observed that the attachment rate was higher at pH 7 than pH 4 or pH 9 in tap water, but lower at pH 7 than pH 4 or pH 9 in distilled water. Attachment of sonicated endospores was higher at pH 7 than pH 4 or pH 9 in distilled water and tap water. The sonicated endospores attached in higher number per J2 in tap water than in distilled water (Davies et al., 1988). Recent studies reveal that the endospore surface has a net negative charge, which was greatest at neutral pH and was reduced with a change of pH away from neutral (Afolabi et al., 1995). Electrostatic forces between the nematode cuticle and the endospore surface oppose attachment because the charges on nematode cuticle also were negative. Reasons for the pH effects remain unclear (Afolabi et al., 1995).

Environmental Survival

Little is known about the long-term survival of endospores of *P. penetrans* in soil. Laboratory studies have shown that *P. penetrans* endospores resist various chemicals and environmental conditions. In the laboratory, endospores of *Pasteuria* sp. were viable for a

period of more than 1 year at 10 to 30 °C and at room temperature (18.5 - 36 °C) (Mani, 1988). Williams et al. (1989) observed that endospores of *P. penetrans* showed properties similar to those of other endospore-forming bacteria in regard to various physical and chemical treatments. The endospores did not take up some stains, were resistant to desiccation and sonication and showed extrusion of endospore contents on prolonged exposure to 0.1% KMnO₄ in 0.3 N HNO₃. Infectivity of *P. penetrans* endospores was reduced after heating at 100 °C for 5 minutes, but endospore attachment was not markedly affected by heating at 100 °C for 15 minutes (Williams et al., 1989). Another test revealed that endospore attachment occurred at up to 80 °C, but infection did not occur at up to 80 °C (Dutky and Sayre, 1978).

The mechanisms for the long-term survival of bacterial endospores include lack of high-energy compounds (ATP and NADH) in the endospore; high content of 3-phosphoglycerate (3PGA), dipicolinic acid (DPA), and divalent cations (Ca²⁺, Mg²⁺, and Mn²⁺); dormancy of enzymes; dehydration of endospore protoplasm; and presence of a thick cortex and coat (Setlow, 1994). The heat resistance can be predicted from the optimal growth temperature of the bacterium, endospore protoplasm water content, total and specific mineral content, temperature of sporulation, and cortex size (Gerhardt and Marquis, 1989). The chemical resistance is related to the impermeability of the protoplasm membrane and endospore coat layers (Setlow, 1994). Endospores of some bacteria can survive in soil for a period of several hundred years to up to 9,000 years (Nilsson and Renberg, 1990; Setlow, 1994). So far, the revealed ultrastructure, morphology, sporogenesis, and chemical properties of endospores of *Pasteuria* spp. are similar to those of other endospore-formers (Bird et al., 1990; Chen et al., 1997a; Williams et al., 1989). Endospores of *P. nishizawae* have thinner cortex and coat layers than *P. penetrans* and also are more sensitive to chemical and heat damage than *P. penetrans* (Nishizawa, 1989). Therefore, it would not be surprising if endospores of *Pasteuria* spp. have prolonged survival in soil.

Natural Enemies

Natural enemies of *P. penetrans* endospores in soil have not been reported. Since the endospores are resistant to various environmental conditions, the survival in soil may largely be affected by biotic factors. A bacterial organism has been observed attached to the central core of endospores when the endospores attach to the cuticle of J2 of *Meloidogyne* spp. (pers. obs.). Whether or not the bacterium is a parasite remains unknown. Apparently, more research should be directed to this important area for understanding the environmental fate of endospores in soil.

Pasteuria penetrans as a Biological Control Agent

Biological Control Potential

Pasteuria penetrans is a very promising biological control agent against root-knot nematodes. The role of *P. penetrans* in suppressing plant-parasitic nematodes has been tested on many crops mostly in greenhouse pots (Table 1.3). *Pasteuria penetrans* suppressed *Meloidogyne* spp. on egg plant, tomato, wheat, tobacco, soybean, bean, pepper, hairy vetch, cucumber, peanut, rye, chickpea, kiwi, grape, brinjal, mung, and okra. Some isolates of *Pasteuria* spp. have been reported to suppress *H. avenae* and *H. zae* on unknown crops (Bhattacharya and Swarup, 1988), *Belonolaimus longicaudatus* on bermudagrass turf (Giblin-Davis, 1990), *H. elachista* on rice (Nishizawa, 1987), and *H. cajani* on cowpea (Singh and Dhawan, 1994).

Two unsuccessful cases involved isolates of *Pasteuria* spp. suppressing *Meloidogyne* spp. on sugarcane (Spaull, 1984) and *Helicotylenchus lobus* on turfgrass (Ciancio et al., 1992). On turfgrass, there was no relationship between the population density of *Helicotylenchus lobus* and the percentage of nematodes with endospores in soil naturally infested with *Pasteuria* sp. (Ciancio et al., 1992). A survey in sugarcane fields in South Africa revealed that populations of

Table 1.3. Experiments using *Pasteuria penetrans* for the control of plant-parasitic nematodes.

Nematode and host	Summary of results	Source
<i>Meloidogyne incognita</i> on tomato	A mixture of <i>M. incognita</i> and <i>P. penetrans</i> endospores resulted in suppression of root galls (81%) and reproduction (97%).	Adiko and Gowen, 1994
<i>M. incognita</i> on tomato, egg plant, and wheat	The inoculation of endospore-encumbered second-stage juveniles (J2) in one crop and re-incorporation of the <i>P. penetrans</i> -infested roots after successive crops was successful and resulted in fewer galls and egg masses on the host plant.	Ahmed, 1990
<i>M. incognita</i> on tomato	<i>Pasteuria penetrans</i> applied 2.5-cm deep in soil depth was more effective than when applied at the soil surface and 5-cm deep.	Ahmed et al., 1990
<i>M. incognita</i> on tomato	Egg masses were reduced by 66% in pots treated with 9,000 endospores/g of soil.	Ahmed and Gowen, 1991
<i>Heterodera avenae</i> and <i>H. zea</i> on unspecified crops	Direct mixing of endospore-infested soil was an effective method of application for the suppression of cyst nematodes.	Bhattacharya and Swarup, 1988
<i>M. incognita</i> , <i>M. javanica</i> , and <i>Meloidogyne</i> sp. on tomato	A nematode suppressive soil from a vineyard was able to suppress egg masses produced on tomato in pot tests and the suppressiveness was removed by autoclaving the soil.	Bird and Brisbane, 1988
<i>M. incognita</i> on tobacco, soybean, and hairy vetch	Yield increases were observed in field plots treated with <i>P. penetrans</i> endospores.	Brown et al., 1985

Table 1.3 Continued

Nematode and host	Summary of results	Source
<i>M. javanica</i> on tomato	In greenhouse pot trials, treatment of <i>M. javanica</i> infested soil with <i>P. penetrans</i> endospores as well as aldicarb (1.5 µg/g) or carbofuran (0.25 µg/g) reduced galling by a factor of 10, much more than by the bacterial or nematicide treatment alone.	Brown and Nordmeyer, 1985
<i>M. incognita</i> on tomato	Root penetration of J2 was inhibited by <i>P. penetrans</i> in laboratory and greenhouse tests.	Brown and Smart, 1985
<i>Meloidogyne</i> spp. on tomato	The application of <i>P. penetrans</i> endospores to soil resulted in significant control of root-knot nematodes.	Channer and Gowen, 1988
<i>Helicotylenchus lobus</i> on turfgrass	No relationship was found between nematode density and the percentage of nematodes with endospores in a soil naturally infested with <i>Pasteuria</i> sp.	Ciancio et al., 1992
<i>M. javanica</i> on tomato	In a factorial experiment, results suggested a density dependent relationship between <i>P. penetrans</i> and <i>M. javanica</i> on tomato.	Ciancio and Bourijate, 1995
<i>M. javanica</i> on tomato	<i>Pasteuria penetrans</i> suppressed galling and egg masses and increased the shoot weight.	Daudi et al., 1990
<i>M. javanica</i> on tomato	<i>Pasteuria penetrans</i> and oxamyl alone or in combination inhibited the production of egg masses.	Daudi and Gowen, 1992
<i>M. incognita</i> on tomato	Tomato root invasion by J2 was reduced by 86% when J2 with ≥ 15 endospores attached were added to soil at high densities (1000 or 3000 J2/plant); numbers of second generation nematodes were reduced by 82 to 93% when J2 with 1 to 15 endospores attached were added to soil.	Davies et al., 1988

Table 1.3 Continued

Nematode and host	Summary of results	Source
<i>M. incognita</i> on tomato	<i>Pasteuria penetrans</i> reduced the motility of J2 and the number of females developed in roots.	Davies et al., 1991
<i>M. incognita</i> on tomato	<i>Pasteuria penetrans</i> reduced root invasion by J2 and resulted in the improved growth of tomato.	De Channer, 1989
<i>M. incognita</i> on tomato	<i>Verticillium chlamydosporium</i> and <i>P. penetrans</i> alone or in combination were tested in a pot experiment; the two biological control agents tended to complement each other and reduced the population density by 92% at the second harvest.	De Leij et al., 1992
<i>M. incognita</i> on tomato, tobacco, soybean, hairy vetch, and pepper	<i>M. incognita</i> was controlled more effectively and yields of host plants were greater when <i>Paecilomyces lilacinus</i> and <i>P. penetrans</i> were applied together in field microplots than when either was applied alone.	Dube and Smart, 1987
<i>Belonolaimus longicaudatus</i> on bermudagrass	Soil infested with a <i>Pasteuria</i> sp. endospores was not suppressive to <i>B. longicaudatus</i> on 'Tifgreen II' bermudagrass in the first 6 months, but caused a significant decrease of the nematode after 1 year in a greenhouse study.	Giblin-Davis, 1990
<i>M. incognita</i> and <i>M. javanica</i> on tomato and cucumber	<i>Pasteuria penetrans</i> applied alone and with oxamyl at reduced dosage on tomato and cucumber caused lower galling incidence, reduced nematode egg production, and resulted in fewer <i>M. javanica</i> and <i>M. incognita</i> juveniles at the end of the crop.	Gowen and Tzortzakakis, 1994

Table 1.3 Continued

Nematode and host	Summary of results	Source
<i>M. javanica</i> on tomato	<i>Pasteuria penetrans</i> reduced the multiplication of the nematode and increased the weight of shoot and root of tomato.	Jaya Raj and Mani, 1988
<i>M. javanica</i> on tomato	<i>Pasteuria penetrans</i> and <i>Paecilomyces lilacinus</i> applied alone enhanced all plant growth characteristics, with <i>P. penetrans</i> being the more effective; combined application of the two organisms was even more effective and increased dry weight of the shoot and lowered soil J2 populations.	Maheswari and Mani, 1988
<i>M. javanica</i> on tomato	Application of <i>P. penetrans</i> in combination with aldicarb, carbofuran, isazofos, phorate, and sebufos improved plant growth and reduced root galling. Such effects were more than additive when compared with their individual effects.	Maheswari et al., 1987
<i>M. javanica</i> on tomato	<i>Pasteuria penetrans</i> as well as four types of oil cakes reduced nematode infestation and improved plant growth, but the combination treatments were significantly superior; <i>P. penetrans</i> applied in combination with neem cake was the best treatment, giving 75% reduction in final J2 populations.	Maheswari et al., 1988
<i>Meloidogyne</i> spp. on tomato	<i>Pasteuria penetrans</i> reduced motility of J2 through soil.	Mankau and Prasad, 1977
<i>M. arenaria</i> on tomato	A soil infested with <i>P. penetrans</i> that was suppressive to <i>M. arenaria</i> on peanut was tested in a greenhouse; reductions of root penetration of J2, root galling, and nematode reproduction were observed.	Minton and Sayre, 1989

Table 1.3 Continued

Nematode and host	Summary of results	Source
<i>Heterodera elachista</i> on rice	<i>Pasteuria</i> sp. suppressed the nematode population after 4 years of exponential increase of the nematode in a reclaimed area.	Nishizawa, 1987
<i>M. arenaria</i> on peanut, rye, and vetch	<i>Pasteuria penetrans</i> populations developed over years in different cropping systems; 3 years after the initial inoculation of endospore-encumbered J2, the peanut yield was increased in plots treated with <i>P. penetrans</i> .	Oostendorp et al., 1991a
<i>M. acronema</i> on tomato	<i>Pasteuria</i> sp. infection of juvenile stages, males, and females resulted in almost complete destruction of nematode cultures in a greenhouse.	Page and Bridge, 1985
<i>M. incognita</i> on tomato	<i>Pasteuria penetrans</i> reduced root penetration by J2, gall formation, and reproduction of the nematode.	Sekhar and Gill, 1990
<i>M. incognita</i> on tomato	Application of <i>P. penetrans</i> and carbofuran, individually or in combination, reduced gall formation and improved growth of tomato in a pot experiment.	Sekhar and Gill, 1991
<i>M. javanica</i> on chickpea	<i>Pasteuria penetrans</i> reduced root galling by 81% and 58% in two greenhouse tests.	Sharma, 1992
<i>H. cajani</i> on cowpea	<i>Pasteuria penetrans</i> reduced root penetration of J2; the final cyst formation and J2 population was reduced by 87% and 199%, respectively, at a level of > 40 endospores /J2.	Singh and Dhawan, 1994

Table 1.3 Continued

Nematode and host	Summary of results	Source
<i>Meloidogyne</i> spp. on sugarcane	Observations in sugarcane fields in South Africa revealed that <i>P. penetrans</i> infected females of <i>Meloidogyne</i> spp. occurred more frequently in coarse than fine textured soils; populations of <i>Meloidogyne</i> spp. were generally greater in fields infested with <i>P. penetrans</i> than in noninfested fields and data collected from one field showed that the level of parasitism was greater at higher densities of the host.	Spaull, 1984
<i>M. javanica</i> on tomato and grape	When tomato root material containing endospores was incorporated into nematode-infested soil, root galls, and soil population of J2 at harvest were reduced by <i>P. penetrans</i> ; numbers of J2 penetrating roots decreased with increasing endospore concentration and distance that J2 moved in soil; in pot experiments with grapes, there were fewer J2 in vineyard soil infested with <i>P. penetrans</i> than without <i>P. penetrans</i> .	Stirling, 1984
<i>M. incognita</i> and <i>M. javanica</i> on tomato and cucumber	Root galling and egg masses on tomato were reduced in plots treated with <i>P. penetrans</i> and oxamyl; the efficacy of the parasite was enhanced by oxamyl applications; when a solarization treatment was included, root galling, egg masses and J2 in soil were reduced after growing cucumber for 10 weeks in treatments with <i>P. penetrans</i> , oxamyl, and solarization; in this case the efficacy of <i>P. penetrans</i> was enhanced after oxamyl application and probably in solarized soil.	Tzortzakakis and Gowen, 1994

Table 1.3 Continued

Nematode and host	Summary of results	Source
<i>M. incognita</i> on tomato	In three greenhouse experiments, <i>P. penetrans</i> reduced root gall indices, numbers of J2 in soil, and egg masses on roots.	Vargas et al., 1992
<i>M. incognita</i> , <i>M. arenaria</i> , and <i>M. hapla</i> on kiwi	In a survey of root-knot nematodes and <i>P. penetrans</i> in a kiwi orchard, the number of females per gram of roots was positively correlated to the percentage of J2 with <i>P. penetrans</i> ; parasitized females also were correlated to percentage of J2 with endospores attached.	Verdejo-Lucas, 1992
<i>M. javanica</i> on grape	Oxamyl and phenamiphos applied directly to soil under drippers on grapevines reduced abundance of J2 and the rate of <i>P. penetrans</i> infection of J2 for periods of ≤ 2 months; soil solarization using clear polyethylene increased the rate of <i>P. penetrans</i> infection for at least 10 months but did not reduce abundance of <i>M. javanica</i> .	Walker and Wachtel, 1989
<i>M. incognita</i> and <i>M. javanica</i> on tobacco	In a nematode suppressive soil in Florida, <i>P. penetrans</i> reduced root galls, egg masses, and numbers of eggs of both <i>M. incognita</i> and <i>M. javanica</i> on tobacco.	Weibelzahl-Fulton et al., 1996
<i>Meloidogyne</i> spp. on brinjal and mung	Application of <i>P. penetrans</i> and <i>Paecilomyces lilacinus</i> enhanced plant growth parameters, such as shoot and root weight and length, in brinjal and reduced root gall indices both on brinjal and mung when these organisms were used individually or in combination.	Zaki and Maqbool, 1990

Table 1.3. Continued

Nematode and host	Summary of results	Source
<i>M. incognita</i> on okra	Efficacy of <i>P. penetrans</i> with two soil fungi, <i>Paecilomyces lilacinus</i> and <i>Talaromyces flavus</i> , and a bacterium, <i>Bacillus subtilis</i> , against <i>M. incognita</i> on okra was tested in pots; application of biological control agents, used individually or in combination with <i>P. penetrans</i> , enhanced plant growth parameters, such as length and weight of shoots, and reduced root gall indices on okra.	Zaki and Maqbool, 1991
<i>M. javanica</i> on okra	Application of root materials containing endospores of <i>P. penetrans</i> into a nematode infested soil reduced root-knot nematode infection and increased length and fresh weights of shoots and roots of okra plants as compared to the control.	Zaki and Maqbool, 1992

Meloidogyne spp. were generally larger in fields infested with *P. penetrans* and that the level of parasitism was greater at higher densities of the nematodes (Spaull, 1984). Both studies were surveys, so the findings are circumstantial. Fully designed experiments are needed to elucidate the role of *Pasteuria* spp. in suppressing nematodes.

Mode of Action

Pasteuria penetrans reduced the number of J2 penetrating roots (Brown and Smart, 1985; Davies et al., 1988; Sekhar and Gill, 1990), number of females in root (Davies et al., 1991), female fecundity (Bird, 1986; Bird and Brisbane, 1988), number of J2 in soil (Davies et al., 1988), and number of eggs on roots (Ahmed and Gowen, 1991; Bird and Brisbane, 1988; Weibelzahl-Fulton et al., 1996). The J2 movement and motility were reduced and the ability to locate the host roots was affected when J2 were encumbered with endospores (Davies et al., 1991; Mankau and Prasad, 1977). Kinesis of J2 also was affected when J2 had at least one endospore attached (Chen et al., unpubl.).

Objectives

During the past 5 decades, agriculturists have depended heavily on the application of nematicides and fumigants for managing practically all nematode diseases. The strong reliance on chemicals has been detrimental to the environment and to the development of alternative methods of management. Although progress is continually being made on breeding crops for resistance to nematodes, peanut cultivars resistant to *M. arenaria* have not been developed. Biological control is an attractive alternative to chemical management. A number of fungi, bacteria, and viruses are noted as antagonists of nematodes. One group of antagonists, *P. penetrans*, appears to have great potential as biological control agents of nematodes. Recent results with *P. penetrans* that are most encouraging include 1) excellent control of root-knot

nematodes in greenhouse and in field microplot tests, 2) recognition that *P. penetrans* plays an active role in nematode suppressive soils, and 3) possible cultivation of *P. penetrans* in artificial media.

The objectives of this research project were: 1) to determine the effects of *P. penetrans* on the population changes of *M. arenaria* on peanut; 2) to determine the number of *P. penetrans* endospores required for suppressing *M. arenaria* on peanut; 3) to determine the effects of nitrogen on the mass production of *P. penetrans* in a greenhouse culture system; 4) to develop a method for rapidly quantifying numbers of endospores attached on J2; and 5) to investigate the ultrastructure, morphology, and sporogenesis of *P. penetrans* with the aid of scanning and transmission electron microscopy.

Chapter 2 QUANTIFICATION OF ENDOSPORES IN TOMATO ROOT MATERIAL

Introduction

Pasteuria penetrans (Thorne) Sayre & Starr, an obligate mycelial and endospore-forming bacterial parasite of root-knot nematodes, has shown potential as a biological control agent of plant-parasitic nematodes (Dickson et al., 1994). It occurs in nematode suppressive soils (Dickson et al., 1992) and has suppressed nematodes in greenhouse (Brown et al., 1985; Dube and Smart, 1987) and field microplot experiments (Oostendorp et al., 1991a). Endospores of *P. penetrans* attach to the cuticle of second-stage juvenile (J2) of *Meloidogyne* spp. in soil. Apparently infection occurs after the J2 enters a plant root, where parasitized nematodes are able to develop into females but are incapable of reproduction (Bird and Brisbane, 1988). The females and sometimes males (Hatz and Dickson, 1992) become filled with endospores of the bacterium, which are eventually released into the soil upon host disintegration. Thus, the only life stage of *P. penetrans* for practical application in soil is the mature endospore.

Pasteuria penetrans has not been grown on artificial media (Bishop and Ellar, 1991). The parasite must be reared on *Meloidogyne* spp., which in turn must be reared on susceptible plants (Sharma and Stirling, 1991) or in a nematode-excised root system (Verdejo and Jaffee, 1988). Generally, endospores of *P. penetrans* are mass-produced in root-knot nematodes on tomato plants. The roots of the plants are air-dried and ground into a fine powder (Sharma and Stirling, 1991). Counting of endospores is nearly impossible in such preparations (Stirling et al., 1990). The number of endospores applied to the soil has been presented in either grams of root

inoculum per kilogram of soil (Dube and Smart, 1987) or as the number of endospores attached per juvenile (Oostendorp et al., 1990). These methods lack accuracy and precision because the number of endospores per unit of root inoculum varies significantly among different preparations of inocula or among inocula from different laboratories (Sharma and Stirling, 1991).

In this study, six methods for determining endospore concentrations in root material are described and compared. The objectives were to develop accurate, precise, and convenient methods for quantifying endospores in root material. Such methods are useful in field evaluations of *P. penetrans* for managing root-knot nematodes.

Materials and Methods

Nematode isolate: An isolate of *Meloidogyne arenaria* (Neal) Chitwood race 1 from Levy County, Florida, was used in this experiment. The isolate was cultured on tomato (*Lycopersicon esculentum* Mill. cv. Rutgers) in a steam-pasteurized potting soil. Eggs of *M. arenaria* were extracted from roots in 0.5% sodium hypochlorite for 30 seconds (McClure et al., 1973) and caught on a sieve with 25- μ m-pore openings, rinsed, and placed on Baermann funnels. The roots and plant debris were caught and separated from the eggs by a sieve with 75- μ m-pore openings nested on a sieve with 25- μ m-pore openings. Second-stage juveniles (J2) that hatched on the first day were discarded to avoid using nematodes that may have acquired endospores in the potting soil. The juveniles were collected by decanting the water suspension from Baermann funnels on a sieve with 25- μ m-pore openings, rinsed, and transferred into a beaker. The number of J2 in the suspension was determined by counting five 1-ml aliquots with a compound microscope.

Pasteuria penetrans isolate: An isolate of *P. penetrans* designated P-20 (Oostendorp et al., 1990), derived from *M. arenaria* race 1 from a peanut field in Levy County, Florida, was

propagated on *M. arenaria* race 1 growing on tomato in a greenhouse. Endospore-attached J2 (up to 5 days old) were obtained by a centrifugation attachment method (Hewlett and Dickson, 1993). Potted tomato seedlings (45-60 days old) were inoculated three times with the endospore-attached J2 (1,000-1,500 J2/pot/time) at intervals of 1 week. Averaged over all inoculations, $96 \pm 5\%$ of the J2 had at least one endospore attached, with an average of 4.9 ± 2.0 endospores/J2 among those J2 with endospores. These estimates were made by counting 20 J2/aliquot. The tomato plants were grown in 15-cm-diameter pots and were fertilized weekly with 1 g of 20-20-20 (N-P-K)/pot. Insecticide applications and water were provided as needed. Root systems were harvested 45 to 60 days after the last inoculation, rinsed, and air-dried. The primary root was removed to reduce unnecessary root debris. The dried root systems were chopped into pieces of 2- to 3-cm lengths and mixed thoroughly. The total root material weighed approximately 1 kg. Random samples of approximately 10 g each were taken for enzymatic disruption and enzymatic disruption + flotation methods.

Quantification methods: 1. Enzymatic disruption method. Six random subsamples of 1 g each were taken from the 10 g of root material and incubated in 50 ml of a 12.5% cytolase PCL5 solution (Genencor International, Rochester, NY). After 3 days, the material was decanted onto a sieve with 600- μ m-pore openings nested in a sieve with 75- μ m-pore openings and subjected to a high-pressure spray of water to dislodge the females (Hussey, 1971). Endospore-filled females, conspicuous by an opaque and white appearance when illuminated from above, were hand-picked with a pipette (Fisher Scientific No. 13-678-20A) with the aid of a microscope at $\times 20$. The “healthy” females appeared glossy and somewhat transparent. The females containing endospores were ground in deionized water using a glass tissue grinder. The suspension, containing endospores, was decanted into a flask and diluted to a volume of 50 ml. Concentrations of endospores in the suspensions were determined by counting five 0.1- μ l aliquots using a hemocytometer (Fisher Scientific No. 02-671-10) at $\times 450$.

2. Enzymatic disruption + flotation method. The same method as described above was used to dislodge females. Root debris with endospore-filled females in water were centrifuged at 1,000g for 3.5 minutes in a 50-ml centrifuge tube. The supernatant was discarded and the pellet was mixed with a 36% (w/v) sucrose solution and centrifuged again. Females floating in the sucrose solution were collected and ground in deionized water, and concentrations of endospores were determined.

3. Mortar disruption method. Approximately 900 g of chopped-root material was ground in a Wiley mill (Model 4, Arthur H. Thomas, Philadelphia, PA, Thomas Scientific No. 3375-E10) to a powder that passed through a sieve with 5-mm-pore openings. Six random subsamples of 0.5 g of this powder plus 5 ml deionized water were placed in a 6-cm-diameter mortar and ground with a pestle to a slurry. The slurry was transferred to a 100-ml Erlenmeyer flask and diluted to 100 ml with deionized water. The endospore concentration in the suspension was determined with a hemocytometer at $\times 450$.

4. Machine disruption method. The ground roots from the mortar disruption method (No. 3) were further ground in a Wiley mill (Intermediate Model, Arthur H. Thomas, Philadelphia, PA, Fisher Scientific No. 08-338) into a powder that passed through a sieve with 250- μ m-pore openings. Six random subsamples of 0.5 g each of this powder were separately suspended in 100 ml of deionized water. The endospore concentration in the suspension was determined with a hemocytometer at $\times 450$.

5. Centrifugation bioassay method. The remainder of the six endospore suspensions from the mortar disruption method (No. 3) were used. The suspensions were passed through a sieve with 25- μ m-pore openings to separate the root debris and further diluted by pipetting 1 ml of the suspension into 9 ml of water. Three subsamples from each diluted suspension were taken to determine the endospore concentrations using the centrifugation attachment method (Hewlett and Dickson, 1993). A combination of 0.9 ml of the diluted suspension and 0.1 ml of a 2-day-

old J2 suspension of 290 ± 25 J2/ml was placed in a 1.0-ml microfuge tube and centrifuged at 8,700g for 2 minutes using a Beckman microfuge (Beckman, Palo Alto, CA). Nematodes were removed from the tubes with a pipette and placed in Corning cell wells (Corning, Corning, NY). Numbers of endospores per J2 were determined on 15 nematodes per replicate.

A standard curve was developed to interpret the relationship of numbers of endospores per J2 to endospore concentrations in the suspension. A series of endospore concentrations (500, 1,000, 5,000, 10,000, 50,000, and 100,000 endospores/ml) was prepared by serially diluting a stock endospore suspension. The stock endospore suspension was made from hand-picked females. Endospore attachment of J2 from individual concentrations was determined as described above. Each concentration was replicated three times. The endospore attachment rates per J2 were subjected to regression analysis. Endospore concentrations in the unknown samples were determined by using the regression equation.

6. Incubation bioassay method. Three subsamples from each of the six endospore suspensions that originated in the mortar disruption method (No. 3) were taken to determine the endospore concentrations. A combination of 0.9 ml of the diluted endospore suspension and 0.1 ml of the J2 suspension was placed into a Corning cell well and incubated at 22 °C to 25 °C for 18 hours. Fifteen J2 per replicate were examined for the number of endospores attached.

A similar series of endospore concentrations as indicated in the centrifugation bioassay method (No. 5) was prepared. Three subsamples from each concentration were incubated with J2 at 22 °C to 25 °C to determine the endospore attachment. The results were subjected to regression analysis. The concentrations of endospores in the unknown suspension were determined from the regression equation.

Results and Discussion

Differences were observed among methods tested for determining endospore concentrations of *P. penetrans* in tomato root material ($P \leq 0.05$) (Table 2.1). The estimations from the mortar disruption and machine disruption methods were larger than those of the other four methods ($P \leq 0.05$). The lowest estimation was from the centrifugation bioassay method ($P \leq 0.05$).

Mortar disruption and machine disruption methods provided the most precise and accurate estimations (Table 2.1). These methods allowed the detection of large numbers of endospores in the tomato root material. The coefficients of variation were relatively small. The similarity between the results of these methods indicated that machine grinding of root materials was effective enough to produce a fine powder containing endospores that could be used for field evaluations of *P. penetrans*. Although both methods gave satisfactory results, the mortar disruption method produced a slurry that often hindered viewing endospores in the suspension. This method was useful for handling small quantities of material. Alternatively, the root powder produced by the machine disruption method had a relatively consistent particle size. Endospore suspensions made from this powder appeared to facilitate viewing of the endospores, primarily due to improved light penetration of the suspension. Mortar disruption and machine disruption methods produced large amounts of plant root debris in the endospore suspension. The removal of the primary root, however, reduced approximately half of the plant root debris, thus enabling use of the hemocytometer for counting endospores. The counting was time-consuming, and experience was required to differentiate between endospores and other particles. If the endospore concentrations are low, these methods may not detect the existing endospores.

Table 2.1. Comparison of six methods for quantification of endospore concentrations of *Pasteuria penetrans* from tomato roots (million endospores per gram of root material).

Method ^a	Average	SD	CV (%)
Mortar disruption	83.7 a ^b	11.6	13.9
Machine disruption	79.0 a	9.4	11.9
Enzymatic disruption	32.1 c	3.8	11.8
Enzymatic disruption + flotation	25.8 c	8.4	32.6
Incubation bioassay	47.7 b	10.4	21.8
Centrifugation bioassay	12.7 d	4.3	33.9

^a Mortar disruption and machine disruption methods used a mortar, and a Wiley mill, respectively, to grind root material followed by hemocytometer counting of endospores. Enzymatic disruption and enzymatic disruption + flotation methods used cytolase to dislodge the females followed by hand-picking of females and centrifuge separation of females, respectively. Incubation bioassay and centrifugation bioassay methods used mortar-grinding of root material followed by an incubation bioassay at 22 to 25 °C and centrifugation attachment method, respectively, to detect the endospore concentrations.

^b Numbers followed by different letters are different ($P \leq 0.05$) according to Duncan's multiple-range test.

Sharma and Stirling (1991) described a method similar to the mortar disruption method for quantification of endospores in root material. They detected 100 to 1,200 million endospores/g of root material, which is 3-35 times higher than was detected with the mortar disruption and machine disruption methods in the present study. They, however, did not provide standard deviations, so comparisons of precision cannot be made. Gowen and Channer (1988) used an unknown method (probably the same method of Sharma and Stirling) to estimate endospore concentrations and reported 480 to 1,859 million endospores/root system, which is approximately 15 to 60 times higher than was obtained in the present study. They did not provide standard deviations either. These differences in the reported endospore concentrations may reflect differences in isolates of *P. penetrans*, cultural conditions, or detection methods.

Enzymatic disruption and enzymatic disruption + flotation methods removed much of the plant root debris from the sample. This facilitated the counting of endospores in the suspensions and reduced counting time. Hand-picking of endospore-filled females, however, was tedious. Approximately 3 to 5 hours may be required to pick all of the females from the root debris of a single plant with a dry weight of approximately 2 g. Enzymatic disruption + flotation, on the other hand, was rapid. The enzymatic disruption + flotation method required approximately 20 minutes to separate endospore-filled females from six samples, which was less than 3% of the total time required for the six samples processed by the enzymatic disruption method. The latter, however, had a small coefficient of variation, indicating a high precision of detection. Both methods produced low numbers of endospores (Table 2.1), implying that many females had been lost or ruptured.

Although the specific weight of endospores of *P. penetrans* is reported to be 1.28 g/cm³ (Oostendorp et al., 1991b), 36% sucrose solution (specific weight of 1.14 g/cm³) is sufficient to float the endospore-filled females. Compared to methods described by Pableo (1981) and

Hussey (1971), the enzymatic disruption + flotation method involved fewer steps to separate endospore-filled females from root debris. The enzymatic disruption + flotation method also used a higher concentration of sucrose solution during centrifugation because endospore-filled females are denser than the uninfected females (pers. obs.). Methods used by Pableo (1981) and Hussey (1971), however, are more desirable for obtaining *Meloidogyne* females for biochemical studies. The enzymatic disruption + flotation method was an extension of Hussey's (1971) method for separating endospore-filled females from root debris.

The incubation bioassay method, which detected 47.7 million endospores/g of root material, was approximately half as effective as the mortar disruption and machine disruption methods. In this method nematodes acquired endospores by moving or swimming in the endospore suspension. The movement of nematodes correlates with the endospore acquisition (Stirling et al., 1990). The lower estimation of endospores as compared to the mortar disruption and machine disruption methods probably was due to decreased nematode movement during the incubation process. Degradation of plant root debris at room temperature could have resulted in lower oxygen levels, which reduces nematode activity (Wong and Mai, 1973). The centrifugation bioassay method gave the lowest estimation among the methods evaluated ($P \leq 0.05$) (Table 2.1) and was unsatisfactory. The poor results obtained with this method may be due to plant root debris covering the J2, thus separating them from endospores during centrifugation. The standard curves were developed with 'clean' suspensions prepared with hand-picked females (Fig. 2.1). Transformations based on such curves will underestimate the endospore concentrations in samples containing debris. Thus, standard curves made from 'dirty' suspensions may be needed to improve the estimates of endospore concentrations in practical samples.

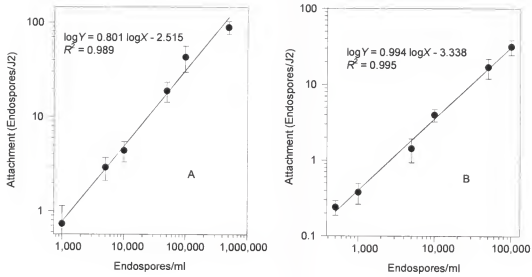


Fig. 2.1. Regression of the number of endospores attached to second-stage juveniles (J2) of *Meloidogyne arenaria* race 1 to endospore concentrations of *Pasteuria penetrans* ($P \leq 0.01$). A) Centrifuge-bioassay method (J2 were centrifuged with endospore suspensions for 2 minutes at 8,700g). B) Incubation-bioassay method (J2 were incubated with endospore suspensions for 18 hours at room temperature [22-25 °C]). Bars show standard deviations. Logarithm scale.

Chapter 3

SOIL APPLICATION OF ENDOSPORES FOR MANAGEMENT ROOT-KNOT NEMATODES ON PEANUT

Introduction

Peanut (*Arachis hypogaea* L.), one of the most important crops grown in the southern United States, is host to a virulent pathogenic nematode, *Meloidogyne arenaria* (Neal) Chitwood race 1 (Minton and Baujard, 1990). In Texas, initial population densities of 8.8 to 16.6 second-stage juveniles (J2) of *M. arenaria* per 100 cm³ of soil were sufficient to cause 10% suppression of yield (Wheeler and Starr, 1987), whereas the damage threshold in Florida was determined to be as low as 1.0 J2/100 cm³ of soil (McSorley et al., 1992). The management of *M. arenaria* relies largely on a combination of methods that includes nematicides and crop rotation (Dickson and Hewlett, 1988a; Minton and Baujard, 1990). Economic considerations and potential environmental hazards of nematicides, however, have created a climate of uncertainty regarding their continued use on peanut. It is also documented that nematicides are often unreliable when nematode population densities are high (Dickson and Hewlett, 1988a), and some successful rotations must remain in place for more than 4 years (Dickson and Hewlett, 1989).

Biological control offers an alternative or supplemental management tactic to chemical or cultural control of nematode pathogens on peanut. It is promising because bacterial parasites and numerous fungal parasites have been observed infecting nematodes in peanut fields in the southern United States (Dickson et al., 1994). One nematode antagonist in particular, *Pasteuria penetrans* (Thorne) Sayre & Starr, has potential for biological control of root-knot nematodes (Dickson et al., 1992; 1994). *Pasteuria penetrans* is an obligate, mycelial, endospore-forming bacterial parasite of

Meloidogyne spp. Endospores of *P. penetrans* attach to the cuticle of the J2 of *Meloidogyne* spp. in soil. Infection occurs after the J2 enters a plant root, where parasitized nematodes are able to develop into females but are incapable of reproduction (Bird, 1986). The females and sometimes males (Dickson et al., 1994) become filled with endospores, which are eventually released into the soil upon host disintegration.

Pasteuria penetrans has been observed in nematode-suppressive soils (Dickson et al., 1994) and successfully tested in greenhouse (Brown and Smart, 1985; De Leij et al., 1992; Stirling, 1984) and field microplot experiments (Brown et al., 1985; Dickson et al., 1992; 1994; Dube and Smart, 1987; Oostendorp et al., 1991a). Attempts to culture *P. penetrans* on artificial media have failed (Bishop and Ellar, 1991). The parasite must be reared on *Meloidogyne* spp., which, in turn, must be reared on susceptible plants or in a nematode-excised root system (Verdejo and Jaffee, 1988). Neither procedure provides production of sufficient inoculum for large-scale applications. Thus, use of this parasite for practical, successful control of the root-knot nematode under normal field cropping conditions has not been accomplished. Also, little information is available on the capability of *P. penetrans* to suppress *M. arenaria* on peanut. The objective of this study was to determine the efficacy of *P. penetrans* on the peanut root-knot nematode at varying soil endospore densities under field conditions.

Materials and Methods

Pasteuria penetrans isolate: An isolate of *P. penetrans* designated P-20 (Oostendorp et al., 1991a), originating from *M. arenaria* race 1 from a peanut field in Levy County, Florida, was propagated on *M. arenaria* growing on tomato (*Lycopersicon esculentum* Mill. cv. Rutgers) in a greenhouse. Second-stage juveniles (J2) up to 5 days old with endospores attached were obtained by a centrifugation attachment method (Hewlett and Dickson, 1993). Tomato plants (45 to 60 days old), which were grown in 15-cm-diameter pots, were inoculated three times with

the endospore-attached J2 (1,000-1,500 J2/pot/time) at intervals of 1 week. Averaged over all inoculations, $96 \pm 5\%$ of the J2 had at least one endospore attached, with an average ($\pm$ SD) of 4.9 ± 2.0 endospores/J2 for those J2 with endospores. These estimates were made by counting 20 J2/aliquot/each inoculation. Tomato plants were fertilized weekly with 1 g of 20-20-20 (N-P-K)/pot. Insecticide applications and water were provided as needed. At 45 to 60 days after the last inoculation, the root systems were harvested, rinsed, air-dried, and ground in a Wiley mill (Intermediate Model, Arthur H. Thomas, Philadelphia, PA) into a powder that passed through a sieve with 250- μ m-pore openings. The root powder weighed approximately 900 g and was stored in a cool room at 13 °C. The endospore concentration was estimated at 79 million endospores/g of root powder by a machine disruption method followed by counting on a hemocytometer (Chen et al., 1996a).

Nematode isolate: An isolate of *M. arenaria* from Levy County, Florida, was cultured on tomato in a steam-pasteurized potting soil. Eggs of *M. arenaria* were extracted from roots in 0.5% sodium hypochlorite for 30 seconds (Hussey and Barker, 1973) and caught on a sieve with 25- μ m-pore openings, rinsed, and placed on Baermann funnels. The roots and plant debris were caught and separated from eggs by a sieve with 75- μ m-pore openings nested on a sieve with 25- μ m-pore openings. Second-stage juveniles that hatched on the first day were discarded to avoid using nematodes that may have acquired endospores in the potting soil. The juveniles were collected by decanting the water suspension from Baermann funnels on a sieve with 25- μ m-pore openings, rinsed, and transferred into a beaker. The number of J2 in the suspension was determined by counting five 1-ml aliquots with the use of a compound inverted light microscope.

Field testing: *Pasteuria penetrans* was evaluated as a biological nematicide over a 2-year period. The experiment had five endospore inoculation levels, 0, 1,000, 3,000, 10,000, and 100,000 endospores/g of soil, with 10 replicates each. Microplots of clay flue liners (Cement Precast Products, Gainesville, FL) were installed in rows of nine microplots with a distance of 0.9 m

between microplots and rows in an Arredondo fine sand soil (94% sand, 1% silt, 5% clay; < 0.1% organic matter; pH 5.6) (McSorley et al., 1992) on the Green Acres Agronomy Farm, University of Florida, Gainesville. The soil profile consisted of a sand ($\geq 90\%$ sand) to a depth of > 1.2 m. The flue liner dimension was 25 cm x 40 cm with a length of 100 cm, and each flue liner was inserted 90 cm deep into the soil. The microplots were disinfected with 1,200 kg of a mixture of 98% methyl bromide and 2% chloropicrin per hectare applied under 0.076-mm-thick polyethylene plastic mulch 3 months before adding inocula.

Meloidogyne arenaria race 1 was introduced into the microplots on 24 May 1994 and increased on tomato roots. Microplots were prepared with a template to make 15 uniformly spaced holes, five each with depths of 7, 14, and 21 cm. A suspension of 25,000 eggs of *M. arenaria* in 300 ml water was sprinkled evenly over the soil and incorporated 7- to 10-cm deep. The hatching rate of the eggs was estimated at 21.6%. One 50-day-old tomato plant (cv. Rutgers) was transplanted into each microplot and fertilized weekly with 0.9 g of 20-20-20 (N-P-K) beginning 7 days after inoculation. After 45 days, the aerial portion of each tomato plant was removed, and the roots chopped into 3- to 5-cm-long pieces before mixing back into the soil of the original microplot. After 2 weeks, soil samples were taken to determine the nematode population densities in each microplot.

On 22 July, the microplots were inoculated with *P. penetrans* endospores. Weighed root powder containing endospores was suspended in 300 ml of water and sprinkled onto the top 20 cm of soil, which was removed from each microplot and mixed in a cement mixer (Stow Manufacturing Company, Oakland, CA) for 2 minutes before placing it back into the original microplot. On 12 August, soil samples were taken to determine the rate of endospores attached per J2. A 10-day-old peanut seedling (cv. Florunner) was transplanted and 1 g of *Rhizobium* spp. (The Nitragin Company, Milwaukee, WI) was dusted onto each microplot. Microplots were drip-irrigated. Insecticides and fungicides were applied as recommended, and each microplot was hand-

weeded as needed. After 125 days, peanuts were harvested. Plant yield, root gall indices, and number of pod galls were recorded. Soil samples were taken to determine the final nematode population density. The root system was chopped into 2- to 5-cm-long pieces and immediately put back into the microplot from which it was removed. The microplots were planted with wheat (*Triticum aestivum* L. cv. Fla 304) as a winter cover crop.

Soil samples consisted of three cores (2.5 cm diameter $\times$ 20 cm deep) taken diagonally across the a microplot. Juveniles were extracted from 100 cm³ soil using centrifugal flotation (Jenkins, 1964) and counted using a compound inverted light microscope. Root galling was indexed on a scale of 0 to 10 based on the percentage of roots galled (0 = no galls on roots; 1 = 1 to 10% roots galled; 2 = 11 to 20% roots galled; ...; 10 = 91 to 100% roots galled) (Barker et al., 1986). Galls on individual pods were counted individually in 1994 and indexed on a scale of 0 to 10 in 1995. Dry weights of foliage and pods were determined by drying at 60 °C for 1 week.

The experiment was continued in 1995. The wheat was cut and the roots mixed into the soil on 2 May. Two weeks later, soil samples were taken and a 10-day-old peanut seedling (cv. Florunner) was transplanted into each microplot. Microplots were managed as in 1994, and data collection was similar. Plants were harvested on 19 September 1995.

Before data analysis, percentage and rating data were transformed by $\arcsin(\sqrt{x})$. Nematode densities and numbers of endospores per J2 were transformed by $\log_{10}(x + 1)$. Dry weight of foliage + pods in 1994 was transformed by $\log_{10}x$ because the dataset had a binomial distribution. Data presented in the text were back-transformed. One microplot in the control was apparently contaminated by *P. penetrans* and thus excluded in statistical analysis. Although regression analysis is usually used with quantitative variables (Mihail and Niblack, 1991), the main objective was to make comparisons among the inoculation levels tested, and therefore Duncan's

multiple-rangetest was used to compare differences among treatment means. Regression analysis also was used, and an equation was provided if the correlation was significant at $P \leq 0.05$.

Results

Pasteuria penetrans reduced the root gall indices, pod gall index, and number of galls on pods and improved peanut production ($P \leq 0.05$) (Fig. 3.1, Table 3.1). The final J2 populations in soil were not affected by *P. penetrans* ($P > 0.05$) (Table 3.1).

1994 Experiment: Initial population densities among treatments before planting peanut were not different ($P > 0.05$) (Fig. 3.2A, Table 3.1). The low number of J2/100 cm³ of soil was due to a high mortality, averaging 94%, after the removal of host tomato. Endospores readily attached to J2 of *M. arenaria* after 3 weeks of exposure of endospores in soil. Numbers of endospores attached to J2 (Y_1) were different among the treatments ($P \leq 0.001$) (Fig. 3.2E, Table 3.1) and correlated with the different levels of endospore inoculations ($X = \log_{10} [\text{inoculation level} + 1]$) ($\log_{10} Y_1 = -2.25 + 0.586X$, $r^2 = 0.867$, $P \leq 0.05$). Root gall index and pod galls per plot were reduced by soil application of endospores ($P \leq 0.05$) (Fig. 3.2C,D; Table 3.1). Compared to the control, root gall index and pod galls per plot were reduced to 40% and 5%, respectively, with 100,000 endospores/g of soil and 80% and 35%, respectively, with 10,000 endospores/g of soil. Low endospore levels of 1,000 and 3,000 endospores/g of soil, when compared to the control, resulted in a slight increase in the root gall index and a decrease in pod galls per plot. This inconsistency was probably due to experimental variation or the low number of endospores attached to J2 at planting (Fig. 3.2E). Dry weights of foliage + pods were increased 41 to 73% by all endospore inoculation levels compared to the control, but there were no differences among the treatments ($P > 0.05$) (Fig. 3.2G, Table 3.1). Final population densities of J2 in soil were not



Fig. 3.1. Biological control of peanut root-knot nematode, *Meloidogyne arenaria* race 1, using the obligate, endospore-forming, bacterial parasite *Pasteuria penetrans*. Endospores were mass produced using a pot culture method and inoculated in field microplots that were preinfested by *M. arenaria*. Photographs were taken in the second year following the inoculation. Upper: The control. Peanut plants had few pods and the pods were severely galled by *M. arenaria*. Below: Microplot was inoculated with 100,000 endospores/g of soil. Peanut plants had few galls on pods.

Table 3.1. Summary of analysis of variance results for effects of *Pasteuria penetrans*, as compared to noninfested controls, on *Meloidogyne arenaria* race 1 and peanut yields.^a

Data measured ^b	1994	1995
J2/100 cm ³ of soil at planting (Pi)	ns	$P \leq 0.01$
J2/100 cm ³ of soil at harvest (Pf)	ns	ns
Root gall index	$P \leq 0.05$	$P \leq 0.001$
Pod gall index	—	$P \leq 0.001$
Pod galls/plot	$P \leq 0.001$	—
Dry weight of foliage + pods (g)	ns	—
Dry weight of foliage (g)	—	$P \leq 0.001$
Dry weight of pods (g)	—	$P \leq 0.01$
Endospores/J2 at planting	$P \leq 0.001$	—
Endospores/J2 at harvesting	$P \leq 0.001$	$P \leq 0.001$
% J2 with attached endospores at harvest	$P \leq 0.001$	$P \leq 0.001$

^aData show significance levels of analysis of variance; ns = not significant at $P \leq 0.05$; — = data not available.

^bPercentage and rating scale were transformed by $\arcsin(\sqrt{x})$. Population density of second-stage juveniles (J2) in soil and number of endospores per J2 data were transformed by $\log_{10}(x + 1)$. Dry weight of foliage + pods in 1994 had a binomial distribution and was transformed by $\log_{10}x$.

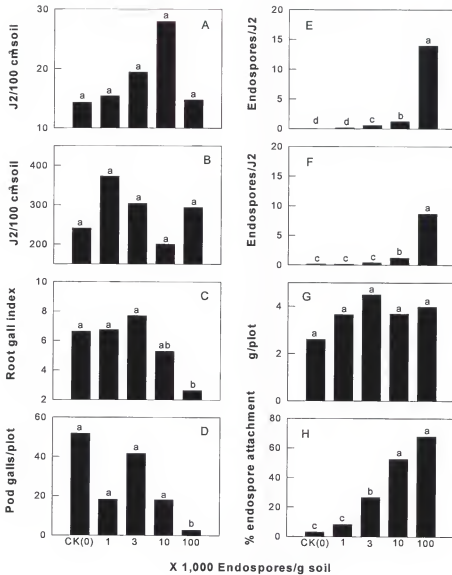


Fig. 3.2. Effect of *Pasteuria penetrans* inoculum levels on *Meloidogyne arenaria* population and peanut yield in 1994. Data are means of 10 replicates, except the control (nine replicates). Same letter on bars indicates no difference ($P > 0.05$) according to Duncan's multiple-range test. For all equations, $X = \log_{10}(\text{endospore inoculum level} + 1)$. A) Population density of second-stage juveniles (J2) in soil at planting. B) Soil J2 population at harvest. C) Root gall index (0 to 10 scale). D) Numbers of galls on pods. E) Number of endospores attached to J2 at planting; $\log_{10} Y = -2.25 + 0.586X$, ($r^2 = 0.867$; $P \leq 0.05$). F) Number of endospores attached to J2 at harvest; $\log_{10} Y = -2.23 + 0.561X$, ($r^2 = 0.910$; $P \leq 0.01$). G) Dry weight of foliage + pods. H) Percentage of J2 with attached endospores at harvest; $Y = -6.92X + 4.23X^2$ ($R^2 = 0.968$; $P \leq 0.01$).

different among treatments ($P > 0.05$) (Fig. 3.2B, Table 3.1). Number of endospores attached to J2 (Y_2) and percentage of J2 with attached endospores at harvest (Y_3) were different ($P \leq 0.001$) and related to the endospore application levels ($\log_{10} Y_2 = -2.23 + 0.561X$, $r^2 = 0.910$, $P \leq 0.01$; $Y_3 = -6.92X + 4.23X^2$, $R^2 = 0.968$, $P \leq 0.01$) (Fig. 3.2F,H; Table 3.1). This indicated that *P. penetrans* persisted in the soil.

1995 Experiment: *Pasteuria penetrans* reduced the overwintered J2 population by planting time in 1995 ($P \leq 0.01$) (Fig. 3.3A, Table 3.1). The J2 in soil (Y_4) were correlated to the endospore inoculation levels ($Y_4 = 4.20 - 1.73X + 0.24X^2$, $R^2 = 0.991$, $P \leq 0.01$) (Fig. 3.3A). After planting peanut, the population densities of J2 in soil showed no differences among treatments ($P > 0.05$) (Fig. 3.3B, Table 3.1). *Pasteuria penetrans* reduced the root and pod gall indices ($P \leq 0.001$) (Fig. 3.3C,D; Table 3.1). Compared to the control, the root and pod gall indices were reduced to 19% and 10%, respectively, for 100,000 endospores/g of soil, and 39% and 18%, respectively, for 10,000 endospores/g of soil. Dry weight of pods was increased 94% and 58% at 100,000 and 10,000 endospores/g of soil, respectively, as compared to the control ($P \leq 0.01$) (Fig. 3.3G, Table 3.1). Dry weight of foliage was increased 75% in microplots with 100,000 endospores/g of soil ($P \leq 0.001$) (Fig. 3.3F, Table 3.1). *Pasteuria penetrans* again persisted in soil in 1995 (Fig. 3.3E,H).

Based on the regression analyses, there was a direct cause and effect of *P. penetrans* on the suppression of pod galls per plot in 1994 and pod gall index in 1995 (Fig. 3.4B,D). Regression analyses indicated that pod galls per plot (Y_5) and pod gall index (Y_6) were negatively correlated with the percentage of J2 attached with *P. penetrans* (Z) ($Y_5 = 46.4 - 0.39Z$, $r = 0.40$, $P \leq 0.001$; $Y_6 = 6.56 - 0.071Z$, $r = 0.57$, $P \leq 0.001$). However, final population densities of J2 in soil in 1994 and 1995 were not correlated with *P. penetrans* levels (Fig. 3.4A,C).

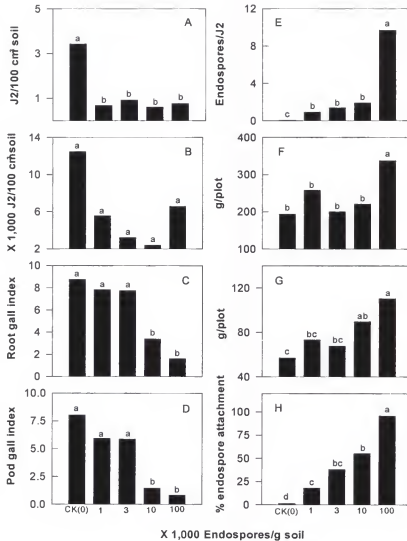


Fig. 3.3. Population changes of *Meloidogyne arenaria* race 1 and *Pasteuria penetrans* in 1995. This was the continued experiment following the endospore inoculation in 1994. Data are means of 10 replicates, except the control (nine replicates). Same letter on bars indicates no difference ($P > 0.05$) according to the Duncan's multiple-range test. For all equations, $X = \log_{10}$ (endospore inoculum level + 1). A) Population density of second-stage juveniles (J2) in soil at planting; $Y = 4.20 - 1.73X + 0.24X^2$ ($R^2 = 0.991$; $P \leq 0.01$). B) Soil J2 population at harvest; $Y = 26916.5 - 8423.29X + 848.25X^2$ ($R^2 = 0.952$; $P \leq 0.05$). C) Root gall index, graded from 0 to 10 based on the percentage of roots galled. D) Pod gall index, graded from 0 to 10 based on the percentage of pods galled. E) Number of endospores attached to J2 at harvest; $\log_{10} Y = -1.34 + 0.438X$ ($r^2 = 0.986$; $P \leq 0.01$). F) Dry weight of foliage. G) Dry weight of pods. H) The percentage of J2 with attached endospores at harvest; $Y = -6.79X + 4.99X^2$ ($R^2 = 0.998$; $P \leq 0.01$).

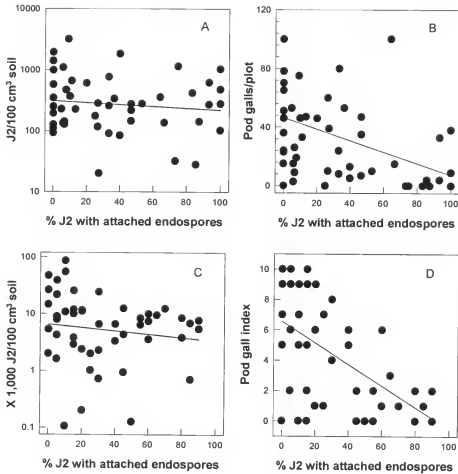


Fig. 3.4. Relationship between the percentage of second-stage juveniles (J2) with attached endospores and population changes of *Meloidogyne arenaria* race 1 on peanut. Data were collected from 49 field microplots in 1994-95 (one microplot in the control was contaminated with *P. penetrans* and excluded in the statistical analysis). A) Relationship between soil J2 population densities at harvest (1994) and the percentage of J2 with attached endospores (1994) at planting; the regression was not significant ($P > 0.05$). B) Relationship between soil J2 population at harvest (1995) and the percentage of J2 with attached endospores (1994) at harvest; the regression was not significant ($P > 0.05$). C) Regression of number of pod galls (1994) to the percentage of J2 with attached endospores (1994) at planting; $Y = 46.4 - 0.390X$, ($r = 0.40$; $P \leq 0.001$). D) Regression of pod gall index (1995) to the percentage of J2 with attached endospores (1994) at harvest; $Y = 6.56 - 0.071X$, ($r = 0.57$; $P \leq 0.001$).

Discussion

Pasteuria penetrans suppressed root knot on peanut in field microplots when applied before the cropping season. The increased yields and reduced galls resulting from adding *P. penetrans* to microplots containing *M. arenaria* demonstrated that *P. penetrans* reduced the damaging effects of *M. arenaria* on peanut. Many researchers have reported similar results with root-knot nematodes on other crops. Brown et al. (1985) claimed a yield increase of 23-24% for tobacco and 38-55% for *Vicia villosa* in a 2-year experiment with *M. incognita*. Dube and Smart (1987) reported a synergistic increase of plant yields on various crops when *Paecilomyces lilacinus* and *P. penetrans* were applied together to control *M. incognita*. Treatment of *M. javanica* infested soil with *P. penetrans* alone (Stirling, 1984) or in combination with aldicarb or carbofuran (Brown and Nordmeyer, 1985) reduced numbers of galls on tomato. The results of the present study evidence that *P. penetrans* was responsible for the yield increase and gall reduction on peanut because they were related to the varied endospore inoculation levels.

The carry-over population densities of J2 in the soil in 1995 was high. It is well established that large numbers of *M. arenaria* can build up on peanut from preplant numbers at or below the detection limits of extraction methods (McSorley et al., 1992; Rodríguez-Kábana et al., 1986; 1982). Densities of *M. arenaria* in soil increase exponentially during the peanut growing season (Rodríguez-Kábana et al., 1986). The highest final density of J2 in soil in the present experiment reached 85,640 J2/100 cm³ of soil, which was much higher than a previously observed “carrying capacity” of 600-6,000 J2/100 cm³ of soil (McSorley et al., 1992; Rodríguez-Kábana and Ivey, 1986). It seems that *M. arenaria* has high reproduction potential on peanut in our deep sandy soil.

Pasteuria penetrans did not suppress the final population densities of J2 in soil. It is possible that the loss of fecundity of parasitized females can be compensated by increased reproduction of healthy females, but the number of J2 in soil at a given time is dependent on so

many factors that a generalization is difficult to make. Another factor that may be addressed is the exponential growth of *M. arenaria*. $Pf = Pi * e^{rt}$ (Rodríguez-Kábana et al., 1986). In this experiment, e^{rt} was estimated to be approximately 3,600. Any difference in Pi will be magnified 3,600-fold in Pf , which makes Pf unpredictable. It is suspected that the overall lower final population densities of J2 in endospore-inoculated microplots in 1995 were due to the lowered Pi , which were caused by *P. penetrans* affecting the overwintering J2. Researchers have reported that *P. penetrans* reduces the motility of J2 in soil (Brown and Smart, 1985; Davies et al., 1991; Mankau and Prasad, 1977; Stirling, 1984). In a petri dish experiment, kinesis of J2 was changed when J2 were attached with any number of endospores (Chen et al., unpubl.). Reduced motility probably leads to a high mortality of J2 in soil because movement apparently is essential to escape predators and unfavorable environmental conditions. Reduction of final J2 population in soil, however, was demonstrated in some experiments (Davies, et al., 1988; De Leij et al., 1992).

Although the final J2 population densities in soil were not significantly affected by *P. penetrans*, the population quality of J2 deteriorated. Percentage of J2 with attached endospores readily increased with the increasing endospore inoculation levels in soil. This suggests that *P. penetrans* may lead to the soil becoming suppressive to *M. arenaria* over the years (Oostendorp et al., 1991a). To date, *P. penetrans* has provided a continuous suppression on a mixed population of *M. incognita* and *M. javanica* in a 7-year monoculture of tobacco (Dickson et al., 1994). Therefore, it appears that endospores of *P. penetrans* are persistent in soil and result in soil becoming suppressive over time (Dickson et al., 1992; Oostendorp et al., 1991a).

Higher yields were obtained at all endospore inoculation levels than in the control. Reduction in galls was greater in plots treated with 10,000 and 100,000 endospores/g of soil than at 1,000 and 3,000 endospores/g of soil. This indicated that at least 10,000 endospores/g of soil may be needed to provide significant suppression of *M. arenaria*. This amount is equivalent to 2×10^{13} endospores/ha in the top 20 cm of soil. The material used in this study contained approximately 7.9

$\times 10^7$ endospores/g of root powder (Chen et al., 1996a); thus, 250 kg of that powder would be required for broadcast treatment of a 1-ha field. Endospore production has been reported as high as 1.2×10^9 (Sharma and Stirling, 1991) and 1.9×10^9 (Gowen and Channer, 1988) endospores/g of root. If those techniques can be duplicated and similar numbers can be obtained for large-scale production, it would reduce the amount of root powder to 17 and 11 kg/ha, respectively, to provide 10,000 endospores/g of soil. Should row treatment be used, the required amount of root powder could be reduced proportionately. When the effectiveness and longevity of the treatment over 2 years are considered, the application of *P. penetrans* has great potential in the near future for the biological control of *Meloidogyne* spp.

Chapter 4

MECHANISMS OF SUPPRESSION OF *MELOIDOGYNE ARENARIA* RACE 1 BY *PASTEURIA PENETRANS*

Introduction

Pasteuria penetrans (Thorne) Sayre & Starr is an obligate mycelial and endospore-forming bacterial parasite of root-knot nematodes (Sayre and Starr, 1985). The first report of an organism resembling *P. penetrans* was made by Cobb (1906), who found numerous highly refractile spores infecting specimens of the nematode *Dorylaimus bulbiferous*. He erroneously viewed these spores as "perhaps monads" of a parasitic sporozoan. This error persisted for nearly 70 years. A more precise but still incorrect placement, *Duboscqia* Perez 1908, was suggested by Micoletzky (1925). Later, Thorne described in detail a new parasite from *Pratylenchus pratensis* (de Man) Filipjev and, on the assumption that the organism was similar to the nematode parasite described by Micoletzky, named it *Duboscqia penetrans* (Thorne, 1940). It was not until the nematode parasite was examined under the electron microscope that its affinities with bacteria rather than protozoa were observed, and it was then named *Bacillus penetrans* by Mankau (1975). Sayre and Starr (1985), who studied the ultrastructure of *B. penetrans*, drew attention to its resemblance to the actinomycete *Pasteuria ramosa* (Metchnikoff, 1888) and subsequently renamed it *Pasteuria penetrans*. Currently, the following three species of *Pasteuria* have been described from plant-parasitic nematodes: *P. penetrans* from *Meloidogyne* spp., *P. thornei* from *Pratylenchus* spp., and *P. nishizawae* from *Heterodera* spp. and *Globodera* spp. (Sayre and Starr, 1989).

Pasteuria penetrans has shown great potential as a biological control agent of root-knot nematodes (Dickson et al., 1994). It occurs in nematode suppressive soils (Dickson et al., 1994) and has suppressed nematodes in greenhouse (Brown and Smart, 1985; De Leij et al., 1992; Stirling, 1984) and field microplot experiments (Brown et al., 1985; Chen et al., 1996b; Dube and Smart, 1987; Oostendorp et al., 1991a; Stirling, 1984). Recently, it was demonstrated that the application of 10,000 endospores of *P. penetrans* per gram of soil effectively suppressed the peanut root-knot nematode on peanut (Chen et al., 1996a; 1996b).

In this study, a two-factor factorial experiment was conducted over a 2-year period under field conditions. The objective was to determine the suppressive relationships between *P. penetrans* infestation levels and *M. arenaria* inoculum densities.

Materials and Methods

Pasteuria penetrans isolate: An isolate of *P. penetrans* designated P-20 (Oostendorp et al., 1990), originating from *M. arenaria* race 1 from a peanut field in Levy County, Florida, was propagated on *M. arenaria* growing on tomato (*Lycopersicon esculentum* Mill. cv. Rutgers) in a greenhouse. Second-stage juveniles (J2) up to 5 days old, with approximately five endospores attached per J2, were obtained by centrifugation (Hewlett and Dickson, 1993). Tomato plants (45- to 60-days old) were inoculated with the endospore-encumbered J2 at a rate of 3,000 J2/pot. The tomato plants were grown in 15-cm-diameter pots and fertilized weekly with 1 g of 20-20-20 (N-P-K)/pot. Insecticide applications and water were provided as needed. Root systems were harvested 45 to 60 days after inoculation, rinsed, and incubated in a 12.5% cytolase PCL5 solution (Genencor International, Rochester, NY) for 3 days. Following incubation, the material was decanted onto a sieve with 600- μ m-pore openings nested in a sieve with 75- μ m-pore openings and subjected to a high-pressure spray of water to dislodge the females (Hussey, 1971).

Endospore-filled females, conspicuous by an opaque and white appearance when illuminated from above, were hand-picked using a pipette with the aid of a microscope at $\times 20$. The females containing endospores were ground in deionized water using a 15-ml glass tissue grinder. The suspension containing endospores was decanted into a flask and diluted to a volume of 50 ml. Concentrations of endospores in the suspensions were determined by counting five, 0.1- μ l aliquots using a hemocytometer at $\times 450$.

Nematode isolate: An isolate of *M. arenaria* race 1 from Levy County, Florida, was cultured on tomato in a steam-pasteurized potting soil. Eggs of *M. arenaria* were extracted from roots using 0.5% sodium hypochlorite (Hussey and Barker, 1973) and caught on a sieve with 25- μ m-pore openings, rinsed, and placed on Baermann funnels. The roots and plant debris were caught and separated from eggs by a sieve with 75- μ m-pore openings nested on a sieve with 25- μ m-pore openings. Second-stage juveniles that hatched on the first day were discarded to avoid using nematodes that may have acquired endospores in the potting soil. Juveniles up to 5 days old were collected by decanting the water suspension from Baermann funnels onto a sieve with 25- μ m-pore openings, rinsed, and transferred into a beaker. The number of J2 in the suspension was determined by counting five, 1-ml aliquots with the use of a compound inverted light microscope.

Field testing: The experiment was conducted in 1994 and 1995. Microplots of clay flue liners were installed in rows of nine separated by a distance of 0.9 m in and between rows in an Arredondo fine sand (94% sand, 1% silt, 5% clay; $< 0.1\%$ organic matter; pH 5.6) (McSorley et al., 1992) at the Green Acres Agronomy Farm, University of Florida, Gainesville. The soil profile consisted of sand ($\geq 90\%$ sand) to a depth of > 1.2 m. The flue liner dimensions were 25 cm $\times$ 40 cm with a length of 100 cm, and each flue liner was inserted 90 cm deep into the soil. The microplots were fumigated in both 1994 and 1995 with 1,200 kg of a mixture of 98% methyl bromide and 2% chloropicrin per hectare applied under a 0.076-mm-thick polyethylene plastic

mulch 3 months before adding inocula.

Meloidogyne arenaria J2 were attached with endospores of *P. penetrans* using centrifugation at 150,000 endospores/ml (Hewlett and Dickson, 1993). After the centrifugation, the suspension was decanted on a 25- μ m-pore sieve to remove the unattached endospores. The endospore-encumbered J2 were then rinsed and transferred into a beaker. Five subsamples, with 20 J2 in each subsample, were taken to determine the number of endospores attached per J2. All J2 had at least one endospore attached, based on the total count of 100 J2 in both 1994 and 1995. The average number of endospores attached per J2 was 3.5 ± 0.3 for 1994 and 5.4 ± 1.0 for 1995. The uninfected J2 were treated similarly except that they were not exposed to endospores. Five hundred J2 in five subsamples taken from the uninfested nematodes were examined at $\times 450$, and no endospore attachment to any of the J2 was observed.

The experimental design was a six $\times$ six factorial with four replications for a total of 144 microplots; six levels of *P. penetrans* infestation and six levels of population densities of *M. arenaria* juveniles were established. The population densities of *M. arenaria* were 0, 40, 200, 1,000, 5,000, and 25,000 J2 per microplot in 1994 and 1995, except that the highest level was 20,000 J2 per microplot in 1995. Six *P. penetrans* infestation levels were established by combining 0, 20, 40, 60, 80, and 100% of endospore-encumbered J2 with uninfested J2 for each population density of *M. arenaria*. The experiment was arranged in a randomized complete block design with a total of four blocks. Microplots were inoculated 20 May in 1994 and 23 May in 1995. The six levels of J2 with the designed percentages of *P. penetrans* infestations were suspended individually in 300 ml of water contained in a 500-ml bottle. Fifteen uniformly spaced holes, five each with depths of 7, 14, and 21 cm, were made in each microplot with a template. The inoculum suspensions were sprinkled uniformly over the soil and into the holes and mixed into the soil by using a potato rake. A 10-day-old peanut seedling (*Arachis hypogaea* L. cv. Florunner) was transplanted into a 3-cm-deep hole in each microplot immediately after the inoculation. After

transplanting, 10 g of inoculum of *Rhizobium* spp. (The Nitragin Company, Milwaukee, WI) were applied into each microplot. Microplots were drip-irrigated and hand-weeded as needed. Insecticides and fungicides were applied as recommended by the University of Florida Extension Service (Whitty et al., 1975). During winter, the microplots were planted with wheat (*Triticum aestivum* L. cv. Fla 304) as a cover crop.

After 125 days, the peanut plants were harvested. Soil samples consisting of three cores (2.5 cm diameter $\times$ 20 cm deep) were taken in each microplot. Juveniles were extracted from 100 cm³ soil using the centrifugal flotation method (Jenkins, 1964) and counted. Endospore attachment at harvest was recorded for 20 J2 for each year of the study. Pod and root galls were counted individually in 1994 and were indexed on a 0 to 10 scale based on the percentage of roots or pods galled in 1995 (0 = no galls; 1 = 1 to 10% galled; 2 = 11 to 20% galled; ...; 10 = 91 to 100% galled) (Barker et al., 1986). Eggs were extracted from roots using 0.5% sodium hypochlorite (Hussey and Barker, 1973) and the number of eggs per root system was determined by counting a 1-ml aliquot from a 20 ml suspension. Dry weights of foliage and pods were determined after drying the tissue at 60 °C for 1 week.

Before data analysis, rating data were transformed with $\arcsin(\sqrt{x/10})$ and nematode densities and numbers of eggs per root system with $\log_{10}(x + 1)$. Means presented in the text were back-transformed. The two-way analysis of variance was used to determine the effects of each factor and the interactions. Regression analysis also was used to establish the relationships between the means and the levels of each main factor. An equation was provided if the correlation was significant at $P \leq 0.05$. Percentage suppression per each 10% increment of the initial *P. penetrans* infestation level was calculated by $b/a \times 10 \times 100\%$, where a is the intercept of the linear equation and b is the slope.

The percentage of suppressiveness was compared with that of a related, previously reported

experiment in which *P. penetrans* endospores were applied directly to soil (Chen et al., 1996b).

Suppression mechanisms of *P. penetrans* against root-knot nematodes are discussed as the relative effectiveness of initial infestation levels of *P. penetrans* at planting versus that of subsequent generations of nematodes that occur during the cropping season.

Results

Results were similar in 1994 and 1995 (Tables 4.1,4.2). In peanut microplots without *P. penetrans* the nematode inoculum density was positively correlated with the number of eggs per root system, J2 per 100 cm³ of soil at harvest, galls on roots or root galling index, and galls on pods or pod galling index in both 1994 and 1995, and root weight in 1995 ($P \leq 0.05$) (Table 4.1,4.2). In peanut microplots infested with *P. penetrans*, the proportion of J2 with attached endospores of *P. penetrans* was inversely correlated with the number of eggs per root system, galls on roots and galls on pods in 1994, and the number of eggs per root system, J2 per 100 cm³ of soil at harvest, root galling index, and pod galling index in 1995 ($P \leq 0.05$) (Table 4.1,4.2).

Based on the regression parameters, the number of eggs per root system, galls on roots, and galls on pods were suppressed by 7.8%, 7.0%, and 8.7%, respectively, for each 10% increment of *P. penetrans* infestation level in 1994 (Table 4.1). The same 10% increment of *P. penetrans* infestation level suppressed the number of eggs per root system, J2 per 100 cm³ of soil, root gall index, and pod gall index by 9.4%, 8.8%, 8.5%, and 8.0% in 1995, respectively (Table 4.2).

Based on two-way analysis of variance, the effects of nematode treatments were significant for the eggs per root system, J2 per 100 cm³ of soil at harvest, galls on roots or root galling index, and galls on pods or pod galling index in both 1994 and 1995 ($P \leq 0.05$). The effects of *P. penetrans* treatments were significant for the number of galls on roots and on pods in 1994, and J2

Table 4.1. Effects of *Pasteuria penetrans* on population densities and galling by *Meloidogyne arenaria* race 1 and on peanut yield per microplot in 1994.^a

Treatment	Levels of treatment	Eggs per root system	J2 per 100 cm ³ soil	Galls per root system	Number of galls on pods	Root weight (g)	Pod weight (g)	Foliage weight (g)
Nematode (J2/plot)	0	9 a ^b	2 a	0.2 a	0.2 a	3.4	40	80
	40	22 a	6 a	7.8 a	1.1 a	5.9	66	145
	200	57 a	7 ab	25.3 a	0.5 a	4.0	42	88
	1,000	229 a	22 b	38.2 a	0.9 a	4.3	46	96
	5,000	697 b	244 c	121.3 b	3.7 b	10.4	38	95
	25,000	1,290 c	489 c	186.0 c	6.7 c	6.9	35	82
Regression parameters ^c	a	0	0	0	0			
	b	0.0553	0.021	0.00814	0.000287			
	r	0.91	0.95	0.84	0.88			
	t-test	**	**	*	*			
<i>P. penetrans</i> (% of J2 with attached endospores)	0	653	18	98.3 a	4.3 a	6.1	47	98
	20	559	38	83.8 ab	2.8 ab	5.8	53	113
	40	332	34	62.1 ab	1.8 b	8.9	42	93
	60	353	22	61.8 bc	2.1 ab	5.0	42	99
	80	283	32	45.5 bc	1.6 b	5.3	44	99
	100	125	19	27.3 c	0.5 b	3.8	38	82
Regression parameters	a	630.4		96.7	3.78			
	b	-4.92		-0.67	-0.0329			
	r	0.96		0.98	0.93			
	t-test	**		***	**			
ANOVA	Block	NS ^d	NS	NS	*	NS	NS	NS
	Nematode	***	***	***	***	NS	NS	NS
	<i>P. penetrans</i>	NS	NS	*	*	NS	NS	NS
	Interaction	NS	NS	NS	NS	NS	NS	NS

^aThis was a six × six factorial experiment. Each mean of the main effect was averaged from 24 microplots. Data of numbers of eggs per root system and numbers of the second-stage juveniles (J2) per 100 cm³ of soil at harvest were transformed with $\log_{10}(x + 1)$ before subjected to ANOVA and presented as back-transformed. Root weight, pod weight, and foliage weight were the fresh weight (g) per microplot and could be transformed to dry weight by multiplying 0.26, 0.57, and 0.26, respectively.

^bMeans followed by different letters are different ($P \leq 0.05$) according to Duncan's multiple-range test.

^cRegression equations are linear ($Y = a + b x$). The t-test was applied to *r*. *, **, *** represent $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$, respectively.

^dNS = not significant at $P > 0.05$.

Table 4.2. Effects of *Pasteuria penetrans* on population densities and galling by *Meloidogyne arenaria* race 1 and on peanut yield per microplot in 1995.^a

Treatment	Levels of treatment	Eggs per root system	J2 per 100 cm ³ soil	Root gall index	Pod gall index	Root weight (g)	Pod weight (g)	Foliage weight (g)
Nematode (J2/plot)	0	5 a ^b	1 a	0 a	0 a	7.9 a	98	308
	40	33 b	3 a	0.2 ab	0.3 a	8.2 a	109	333
	200	84 b	3 a	0.2 ab	0.2 a	8.0 a	96	277
	1,000	561 c	19 b	0.6 b	0.1 a	6.8 a	60	210
	5,000	4,830 d	274 c	4.8 c	3.0 b	14.7 b	88	304
	20,000	14,420 d	740 c	7.2 d	5.4 c	18.6 b	67	253
Regression parameters ^c	a	0	0	0	0	8.26		
	b	0.592	0.038	0.00046	0.00029	0.00056		
	r	0.989	0.991	0.90	0.944	0.92		
	t-test	***	***	**	**	**		
<i>P. penetrans</i> (% of J2 with attached endospores)	0	804	46 ab	3.1 a	1.8 a	12.3	83	276
	20	675	53 a	2.2 ab	1.4 a	11.7	75	258
	40	281	11 bc	1.1 bc	1.1 ab	11.7	104	350
	60	144	26 abc	1.8 ab	1.0 ab	11.2	99	338
	80	134	13 bc	1.1 bc	0.7 ab	11.5	105	306
	100	217	9 c	0.3 c	0.3 b	5.9	53	158
Regression parameters	a	711.2	47.0	2.79	1.74			
	b	-6.71	-0.414	-0.0237	-0.0139			
	r	0.87	0.81	0.90	0.99			
	t-test	*	*	*	***			
ANOVA	Block	NS ^d	NS	NS	NS	NS	NS	NS
	Nematode	***	***	***	***	***	NS	NS
	<i>P. penetrans</i>	NS	*	***	*	NS	NS	NS
	Interaction	NS	NS	NS	NS	NS	NS	NS

^aThis was a six × six factorial experiment. Each mean of the main effect averaged from 24 microplots. Data of numbers of eggs per root system and numbers of the second-stage juveniles (J2) per 100 cm³ of soil at harvest were transformed with $\log_{10}(z + 1)$ before analysis. The root gall index and pod gall index were graded from 0 to 10 based on the percentage of roots and pods galled (0 = no galls; 1 = 1 to 10% galled; 2 = 11 to 20% galled; ...; 10 = 91 to 100% galled). The grading data were transformed with $\arcsin(\sqrt{x/10})$ before subjected to ANOVA and presented as back-transformed. Root weight, pod weight, and foliage weight were the fresh weight (g) per microplot, and could be transformed to dry weight by multiplying 0.30, 0.59, and 0.26, respectively.

^bMeans followed by different letters are different ($P \leq 0.05$) according to Duncan's multiple-range test.

^cRegression equations are linear ($Y = a + bx$). The t-test was applied to *r*. *, **, *** represent $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$, respectively;

^dNS = not significant at $P > 0.05$.

per 100 cm³ of soil at harvest, root galling index, and pod galling index in 1995 ($P \leq 0.05$). There were no interactions between nematode treatments and *P. penetrans* treatments in either year ($P > 0.05$).

Except for peanut root weight in 1995, peanut pod weight, root weight, and foliage weight were not affected by *P. penetrans* or nematode treatments in 1994 or 1995 ($P > 0.05$) (Tables 4.1, 4.2). The peanut root weight correlated with the nematode inoculum levels in 1995 ($P \leq 0.05$) (Table 4.2). The increased root weight with increasing nematode levels in 1995 was due to root galls that contained more root tissue than healthy roots (Table 4.2).

The endospore attachment of J2 at harvest was generally low for both years, averaging over all microplots only 0.05 endospores/J2 in 1994 and 0.14 endospores/J2 in 1995. The percentage of J2 with at least one endospore attached was 3.3% in 1994 and 7.6% in 1995.

Discussion

Pasteuria penetrans suppressed root-knot nematodes on peanut in both years of the study. The suppression was proportional to the *P. penetrans* infestation levels, regardless of the initial population densities of root-knot nematodes. Therefore, the suppression was independent of the nematode population density. This suggested that *P. penetrans* was a reliable biological control agent for managing root-knot nematodes at various population densities.

Neither the nematode inoculum levels nor the *P. penetrans* infestation levels affected peanut growth and yields. The yields, determined by weight, were not a good index because diseased tissue tended to be heavier than healthy tissue. The quality of peanut pods, here presumed to be inversely proportional to the number of galls on pods and pod gall index, was improved by increasing *P. penetrans* infestation levels, but the quality deteriorated with increasing nematode inoculum levels. In a related experiment, both yield increase and quality improvement occurred

when endospores of *P. penetrans* were added directly to microplot soil (Chen et al., 1996b).

The initial infestation level of *P. penetrans* was an important factor in the suppression of root-knot nematodes on peanut. The suppressiveness correlated well with the initial *P. penetrans* levels. *Pasteuria penetrans* did not built up to a suppressive population density in soil during one tested cropping season because most endospore-filled females were removed from the microplots along with root systems, when much of the root system was harvested. This was verified by the low endospore attachment of J2 at harvest in both years. Moreover, the chance of *P. penetrans* infection of subsequent generations of nematodes during the cropping season was probably negligible, either because of the minor endospore build-up in soil or because J2 that hatched from egg masses on root surfaces quickly entered adjacent roots without being exposed to endospores in soil (Stirling, 1984). Reductions of number of galls on pods and reductions in the pod gall index were 8.0% to 8.7% per 10% increment of initial *P. penetrans* levels. Similar results were obtained in a previously reported study of using soil application of *P. penetrans* endospores (Chen et al., 1996b), in which the reduction of pod galls was 8.4% to 10.8% per 10% increment of endospore attachment before the season (Chen et al., 1996b). When soil is treated with *P. penetrans* endospores, the chance of the bacterial infection of subsequent generations of nematodes during the cropping season should be anticipated. Reduction of pod galls in an experiment using soil application of endospores (Chen et al., 1996b), however, was only slightly higher than that in the present study. Thus, the suppressiveness contributed from the *P. penetrans* infection on subsequent generations of nematodes was minor. Again, this demonstrated that the initial infestation level of *P. penetrans* was critical for the biological control of *M. arenaria*. Supposedly the increase in suppressiveness observed in the soil endospore application experiment (Chen et al., 1996b) was contributed to the bacterial infection of subsequent generations of nematodes during the cropping season. Thus, the initial infestation levels can be calculated to have contributed 81% to 95% of the total suppressiveness of the pod galls, while the infection of subsequent generations of nematodes

contributed only 5% to 19%. The limited importance of the infection of subsequent generations of nematodes might result from the short migration distances for J2 that hatched on root surfaces to invade nearby roots (Stirling, 1984).

The population build-up of root-knot nematodes decreased when the initial infestation level of *P. penetrans* was increased. Numbers of eggs per root system in both years and the number of J2 in soil in 1995 were inversely correlated with the initial infestation level of *P. penetrans*. Initial infestation of *P. penetrans* reduced the numbers of J2 invading roots (Stirling, 1984). Nematode reproduction is blocked after the endospore-encumbered J2 entered roots and the bacterium developed in the nematode pseudocoel (Bird, 1986). Because of the importance of the initial *P. penetrans* levels, practices such as solarization, nematicide incorporation, and other integrated pest management measures might be optimized to increase the initial infestation levels of *P. penetrans* for biological control of root-knot nematodes (Brown and Nordmeyer, 1985; Maheswari et al., 1987; Walker and Wachtel, 1989).

Behavior and efficacy of *P. penetrans* do not always conform to general biological control concepts. The most successful examples of biological control of pests have been against insects of perennial crops (Bennett, 1974). Perennial crops provide a stable habitat for both the insect pests and their natural enemies. The vegetational diversity is also important for encouragement and retention of effective populations of natural enemies for insect pests (Bennett, 1974). *Pasteuria penetrans*, however, has successfully suppressed root-knot nematodes on both annual and perennial crops (Stirling, 1984; Chen et al., 1996b). Monoculture and rotation with non-host crops for root-knot nematode management has no obvious adverse effects on *P. penetrans* (Dickson et al., 1994). Population densities of *P. penetrans* increased in monoculture over years (Dickson et al., 1994). Several adaptations of *P. penetrans* may account for the reduced impact of crop habitat and vegetational diversity on its effectiveness as a biological control agent. *Pasteuria penetrans* produces endospores that are dormant in soil. Although it is not known exactly how many years *P.*

penetrans endospores can survive in soil, similar endospores of other bacteria probably survive in soil for more than 1,000 years (Logan, 1994). This is different from the natural enemies of insects that usually require a constant food supply to maintain their population densities. Endospores of *P. penetrans* are highly resistant to heat, desiccation, nematicides, and other adverse environmental conditions (Stirling, 1991). A short period of absence of host crops has little effect on the endospore survival, but may force J2 to move long distances in soil to locate host roots and facilitate the attachment of endospores to the J2 (Stirling, 1984). The endospore dormancy in soil and the endospore resistance to different environments may be the key traits attributing to the success in suppressing root-knot nematodes on both annual and perennial crops under various ecological conditions and crop regimes.

Chapter 5
ESTIMATING INCIDENCE OF ATTACHMENT OF *PASTEURIA PENETRANS*
ENDOSPORES TO *MELOIDOGYNE* SPP. USING TALLY THRESHOLDS

Introduction

Pasteuria penetrans (Thorne) Sayre & Starr is an obligate, mycelial and endospore-forming bacterial parasite of root-knot nematodes (Sayre and Starr, 1985). Endospores of *P. penetrans* attach to the cuticle of the second-stage juveniles (J2) of *Meloidogyne* spp. in soil. The endospores germinate and send a germ tube through the nematode body wall and into the pseudocoel after the J2 enter a plant root and establish a feeding site. The parasitized nematodes are able to develop into females but are incapable of reproduction (Bird, 1986). The females, and sometimes males (Hatz and Dickson, 1992), become filled with endospores, which are eventually released into soil upon host disintegration.

Determining the endospore attachment to J2 is important in evaluating the biological control efficacy of *P. penetrans*. Also, many bioassay projects involve counting the numbers of endospores attached to J2. Endospores of *P. penetrans* are able to attach to any part of the J2 body surface (Stirling, 1991), but the constant movement of nematodes and the contents of their intestines hinder viewing and direct counting under the light microscope.

Recently, binomial sampling became a useful method in estimating population densities of organisms that have a binomial distribution (Binns and Bostanian, 1990; Feng et al., 1993). In binomial sampling, the only information retained from a sample unit is whether or not the organism was present. The percentage of units without the organism is used to estimate the mean density, based on an empirical relationship between mean density and the percentage infestation level.

Binomial sampling usually reduces the time and effort spent estimating the mean density (Binns and Bostanian, 1990). The shortcomings associated with binomial sampling include the requirement of a large number of samples and a higher variance associated with the mean density than for methods involving with direct counting of all individuals (Binns and Bostanian, 1990; Nyrop and Binns, 1991). The application of tally thresholds in binomial sampling has been reported to greatly reduce the variance and thus make binomial sampling more attractive (Binns and Bostanian, 1990; Feng et al., 1993; Nyrop and Binns, 1991). A tally threshold (T) is defined as the maximal number of individuals in a sample unit that may be treated as absent (Binns and Bostanian, 1990; Nyrop and Binns, 1991). For example, when T was set to 5, samples with ≤ 5 organisms were counted as absent. Therefore, a tally threshold sampling using $T = 0$ defaults to the binomial sampling.

Before tally thresholds can be used practically, the relationship between the mean densities (m) and the proportions of no more than a predetermined tally threshold of individuals (P_T) must be determined. This can be done through a theoretical distribution, such as the Poisson (Tippett, 1932) or the negative binomial (Pielou, 1960), or through regression. Little is known about the relationship between the mean number of endospores attached per J2 (mean density) and the proportion of sample units with different tally thresholds. The objectives of this study were to establish the empirical relationships between m and P_T and determine the potential sampling errors associated with the application of the tally thresholds.

Materials and Methods

Data collection: The following three previously reported data sets were used for mathematical analyses: endospore attachment of *P. penetrans* to J2 of *M. arenaria* race 1 in a centrifugal bioassay (Chen et al., 1996a), an incubation bioassay (Chen et al., 1996a), and field experiments (Chen et al., 1996b). In the centrifugal bioassay, endospore-filled females were

hand-picked and ground in deionized water using a glass-tissue grinder. The suspensions were usually diluted in water containing approximately 1 million endospores per ml. A combination of the diluted endospore suspensions and 2-day-old J2 suspensions were placed in a microfuge tube and centrifuged at 8,700g for 2 minutes using a microfuge (Hewlett and Dickson, 1993). Nematodes were removed from the tubes with a pipette and placed in Corning cell wells (Corning, Corning, NY). Numbers of endospores attached to individual J2 were determined on 15 to 31 nematodes per sample with the aid of light microscope at $\times 450$. Seventy samples with a range of endospore attachment from 0.2 to 20.9 endospores/J2 were examined.

In the incubation bioassay, tomato roots containing approximately 80 million endospores/g of root material were ground in a Wiley mill (Model 4, Arthur H. Thomas, Philadelphia, PA) to a powder that passed through a sieve with 5-mm-pore openings (Chen et al., 1996a). A 0.5-g sample of the root powder plus 5 ml of deionized water were placed in a 6-cm-diameter mortar and ground with a pestle to a slurry. The slurry was transferred to a 100-ml Erlenmeyer flask and diluted to 100 ml with deionized water. A combination of 0.9 ml of the diluted endospore suspension and 0.1 ml of a 2-day-old J2 suspension of 290 ± 25 J2/ml was placed into a Corning cell well (Corning, Corning, NY) and incubated at 22 to 25 °C for 18 hours. The number of endospores attached per J2 was estimated on 15 J2. Thirty-three samples were examined and the range of endospore attachment attained was 0.3 to 36.0 endospores/J2.

In the field experiments, the field soil was infested with *P. penetrans* endospores before the cropping season for evaluating its suppressiveness to root-knot nematodes (Chen et al., 1996b). Second-stage juveniles were extracted at harvest from soil using centrifugal flotation (Jenkins, 1964). Numbers of endospores attached per J2 were determined on 15 to 20 nematodes per sample. One hundred eleven samples, with a range of endospore attachment of 0.1 to 28.8 endospores/J2, were examined.

Data from centrifugal bioassay, incubation bioassay, and field experiment were used separately to compute the mean endospore attachment (m), s_m^2 , and the proportion of J2 (P_T) with $\leq T$ (tally threshold) endospores attached; T was set to 0, 1, 2, 3, 4, 5, 8, and 10 endospores/J2. The relationships between $\ln(m)$ and P_T were established for each of the three data sets at different T values.

Data Analysis: For each tally threshold, the empirical relationship between m and P_T was developed using parameters from the linear regression of $\ln(m)$ on P_T ($0 < P_T < 1$):

$$\ln(m) = a' + b' P_T \quad (1)$$

Where m is the mean number of endospores attached per J2; P_T is the proportion of J2 with $\leq T$ endospores attached; and a' and b' are intercept and slope parameters from the regression, respectively. The antilogarithm form of equation 1 is:

$$m = e^{a' + b' P_T} \quad (2)$$

This equation gives an estimate of m for a given P_T obtained in the sample.

The variance $\text{var}(m)$, which is associated with estimation of m from P_T , consists of two components, a prediction variance, $\text{var}_p(m)$, and sampling variance, $\text{var}_s(m)$ (Binns and Bostanian, 1990; Feng et al., 1993; Nyrop and Binns, 1991):

$$\text{var}_p(m) = m^2 s_m^2 (1/N + (P_T - \text{avg}P_T)^2/\text{SSP}_T) \quad (3)$$

$$\text{var}_s(m) = m^2 s_m^2 \quad (4)$$

Where N is the number of data points used to fit equation 1; s_m^2 is the variance of equation 1; $\text{avg}P_T$ is the mean value of the independent variable P_T used in the regression; SSP_T is the sum of the squares of the deviations of the independent variable P_T used in the regression.

To compare the potential sampling errors that would be involved in estimating m from P_T using the empirically determined m and P_T relationships, Karandinos' formula was used (Karandinos, 1976):

$$n = \left(\frac{Z_{\alpha/2} s}{dm} \right)^2 \quad (5)$$

where $Z_{\alpha/2}$ is the standard normal deviate such that $P(Z > Z_{\alpha/2}) = \alpha/2$; d is the predetermined confidence interval half-width (CI) as a proportion of the mean ($d = \text{CI}/m$); and s^2 is the variance estimated for prediction of m , here $s^2 = \text{var}_p(m) + \text{var}_s(m)$. When α is set to 0.05 (95% confidence), then $Z_{\alpha/2}$ equals approximately 2 and the sampling error ($E = d/Z_{\alpha/2}$) is approximately half of the estimated d value. Equation 5 was rearranged and the terms of $\text{var}_p(m)$ and $\text{var}_s(m)$ were incorporated:

$$d = \frac{Z_{\alpha/2}}{m} \sqrt{\frac{\text{var}_p(m) + \text{var}_s(m)}{n}} \quad (6)$$

This equation gave a d value that could actually be attained when an estimate of P_T , obtained in the sample reading and based on a desired sample size n , was used to estimate the corresponding m . It should be noted that d would change with n and P_T in relation to m . The α level used for calculation of d was always 0.05.

The relationships of means (m) and variances associated with the means (s^2) for endospore attachment data from the centrifugal bioassay, incubation bioassay, and field experiments were subjected to analyses by Taylor's power law (Taylor, 1961):

$$\ln(s^2) = \ln(a) + b \ln(m) \quad (7)$$

In Taylor's power law, the relationship between $\ln(s^2)$ and $\ln(m)$ is linear. The slope value, b , indicates the kind of distribution that m may possess. For example, a slope value of greater than 1 indicates a contagious distribution (Taylor, 1961). The tally threshold sampling requires that the mean (m) has a contagious distribution (Binns and Bostanian, 1990; Feng et al., 1993; Nyrop and Binns, 1991).

Results and Discussions

The results of Taylor's power law analyses are shown in Fig. 5.1. The variances (s^2) regressed better to the means of data from the incubation bioassay and field experiments, yielding high values of r^2 , than to the centrifugal bioassay. The slopes from all three data sets were > 1 , indicating a contagious distribution of the mean, regardless of different methods used for endospore attachment ($P \leq 0.01$). The slopes from data attained from the incubation bioassay and the field experiment were 1.6, which was greater than the slope of 1.3 attained with the centrifugal bioassay ($P \leq 0.01$). The means from the centrifugal bioassay had a less contagious distribution than the other two, possibly because of the centrifugal technique having a homogenous endospore concentration and the centrifugal force causing endospore attachment. In all cases, Taylor's intercepts $\ln(a)$ were > 0 (Fig. 5.1.), indicating that the ratio of s^2/m , which was equal to am^{b-1} ($a > 1, b > 1$), was > 1 and changed with m for each of the data sets. Therefore, a logarithm transformation should be done before the analysis of variances and means separation test are conducted (Southwood, 1978).

Results from the linear regression of $\ln(m)$ on P_T are listed in Table 5.1. The regression was not significant for $T = 10$ for the field experiments data set, and the results were thus omitted. Generally, Equation 1 fit the data sets well with coefficients of determination (r^2) of approximately 0.8 for most of the tally thresholds concerned, regardless of endospore attachments from the centrifugal bioassay, incubation bioassay, and field experiments. The variances of Equation 1 (s_m^2) tended to decrease with increasing T values for all three occasions. The variances also were greater for endospore attachment incidences from field experiments than those from the incubation bioassay and centrifugal bioassay (Table 5.1). Under field conditions,

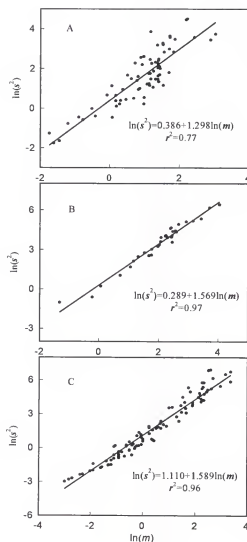


Fig. 5.1. Analysis of the relationships between the means (m) and the variances (s^2) associated with m using Taylor's power law. The means were the number of *Pasteuria penetrans* endospores attached to the second-stage juveniles of *Meloidogyne arenaria* race 1. A) Endospore attachment from a centrifugal bioassay. B) Endospore attachment from an incubation bioassay. C) Endospore attachment from field experiments.

Table 5.1. Parameters from linear regression of the number of endospores attached per second-stage juvenile ($\ln(m)$) as a proportion of juveniles with $\leq T$ endospores attached (P_T) at different T values.^a

T	N^b	a	b	r^2	s_m^2 ^c	SSP_T ^d	m ranges	$\text{avg}P_T$ ^e
Endospore attachment from the centrifugal bioassay								
0	42	1.4152	-3.1962	0.84	0.1273	2.6802	0.2-4.8	0.2990
1	64	1.9203	-2.7149	0.83	0.1076	4.2924	0.2-9.3	0.3779
2	60	2.2311	-2.2555	0.73	0.1007	3.1751	0.4-9.3	0.5203
3	57	2.5065	-2.1201	0.68	0.0853	2.2571	0.9-9.3	0.6480
4	54	2.9478	-2.3389	0.75	0.0755	2.1780	1.1-20.9	0.7215
5	49	2.9858	-2.1742	0.71	0.0740	1.8028	1.1-20.9	0.7565
8	33	3.3366	-2.1758	0.79	0.0616	1.5118	2.1-20.9	0.8203
10	26	3.3801	-2.1473	0.81	0.0634	1.3835	2.1-20.9	0.8180
Endospore attachment from the incubation bioassay								
0	21	2.3833	-4.8348	0.81	0.1948	0.6768	0.3-11.6	0.1810
1	26	2.7040	-3.9780	0.87	0.1250	1.2891	0.3-14.4	0.2538
2	26	2.7605	-2.8491	0.74	0.1126	0.9372	1.1-14.4	0.2855
3	28	2.9342	-2.5732	0.79	0.1033	1.4879	1.1-23.4	0.3535
4	27	2.9657	-2.2376	0.75	0.0847	1.2851	2.1-23.4	0.3881
5	27	3.0922	-2.2197	0.76	0.0830	1.3146	2.1-23.4	0.4483
8	26	3.3248	-2.1519	0.83	0.0471	1.1811	2.9-23.4	0.5459
10	25	3.5408	-2.1616	0.86	0.0356	1.1053	3.7-36.0	0.5783
Endospore attachment from field experiments								
0	102	3.0253	-5.5510	0.84	0.4405	7.5461	0.1-23.7	0.5805
1	83	3.2173	-4.3385	0.85	0.2784	6.5330	0.2-23.7	0.6014
2	70	3.4490	-3.9330	0.83	0.2397	4.9922	0.3-23.7	0.6277
3	63	4.0946	-4.3654	0.83	0.2292	3.4860	0.3-28.8	0.6643
4	52	3.9604	-3.7388	0.79	0.2052	2.7392	0.7-28.8	0.6559
5	49	4.1211	-3.6924	0.75	0.2149	2.2544	0.8-28.8	0.6823
8	41	4.0781	-3.1223	0.68	0.1880	1.6388	1.3-28.8	0.6885

^aThe linear equation is $\ln(m) = a + b P_T$, where m is the mean number of endospores attached per J2; P_T is the proportion of J2 with $\leq T$ endospores attached; all regression equations were significant at $P \leq 0.001$; T is the tally threshold defined as the maximal number of individuals in a sample unit that may be treated as absent; and a and b are intercept and slope parameters from the regression analysis, respectively.

^b N is the number of data points used to fit the equation.

^c s_m^2 is the variance of the equation.

^d $\text{avg}P_T$ is the mean value of the independent variable P_T used in the regression.

^e SSP_T is the sum of the squares of the deviations of the independent variable P_T used in the regression.

the age structure of J2 and the endospore concentration distribution in soil probably were highly variable. Many environmental conditions and ecological factors could affect the attachment incidence of *P. penetrans* to *Meloidogyne* spp. (Bird et al., 1989; Chen et al., 1996a; Hatz and Dickson, 1992; Stirling et al., 1990). Second-stage juveniles with different numbers of endospores attached may also have different fates in soil. The non-significant regression at $T = 10$ under field condition suggested that the mortality of J2 with > 10 endospores attached probably increased abruptly, hence yielding a randomized P_T that no longer correlated with the change of $\ln(m)$.

The m range increased with increasing T values, because P_T was preset at $P_T > 0$ and $P_T < 1$. Data pairs of $\ln(m)$ and P_T with $P_T = 1$ were excluded from regression analysis to reduce the bias. When estimations were based merely on the proportion of J2 with no endospores attached ($T = 0$), the variances were greater than those for the other T values (Table 5.1). Thus, choosing a rational T value would give rise to a precise estimation of m , along with a desirable detection range of m . Accordingly, in Table 5.1, T values of 0, 1, 8, and 10 were important for estimating the endospore attachment (m) from P_T in the centrifugal bioassay and incubation bioassay, whereas 0, 1, 2, and 3 were important for field experiment data. These T values were associated with both high coefficients of determination (r^2) and different m ranges that were ideal for detecting both low and high numbers of endospores attached per J2.

Half-widths of 95% confidence intervals (d) (Eq. 6) varied from 0.04 to 0.37 among the T values for data sets from the centrifugal bioassay, incubation bioassay, and field experiments (Table 5.2). The d values also were variable with changing of P_T in relationship to $\text{avg}P_T$ (Eq. 3); hence, they are presented as a range of d . The d value for a given T value would be reduced by increasing the sample size (n). High d values were associated with the estimation of m from P_T in the data set from the field experiment, whereas low d values were associated with the

Table 5.2. Confidence interval half-widths (d) associated with the estimation of number of endospores attached per second-stage juvenile (m) from the proportion of juveniles with $\leq T$ endospores attached (P_T) at different T values with different sample sizes (n).^a

T^b	Ranges of confidence interval half-widths				m ranges
	$n = 15$	$n = 25$	$n = 50$	$n = 100$	
Endospore attachment from the centrifugal bioassay					
0	0.20-0.22	0.15-0.17	0.10-0.11	0.08-0.08	0.2-4.8
1	0.18-0.19	0.14-0.14	0.09-0.10	0.07-0.07	0.2-9.3
2	0.18-0.19	0.14-0.14	0.09-0.10	0.07-0.07	0.4-9.3
3	0.16-0.18	0.12-0.14	0.08-0.09	0.06-0.07	0.9-9.3
4	0.15-0.17	0.11-0.13	0.08-0.09	0.05-0.06	1.1-20.9
5	0.15-0.17	0.11-0.13	0.08-0.09	0.05-0.06	1.1-20.9
8	0.14-0.17	0.10-0.12	0.07-0.08	0.05-0.06	2.1-20.9
10	0.14-0.17	0.11-0.13	0.07-0.09	0.05-0.06	2.1-20.9
Endospore attachment from the incubation bioassay					
0	0.25-0.35	0.19-0.26	0.13-0.18	0.09-0.12	0.3-11.6
1	0.20-0.24	0.15-0.18	0.10-0.12	0.07-0.08	0.3-14.4
2	0.19-0.23	0.14-0.17	0.09-0.12	0.07-0.08	1.1-14.4
3	0.18-0.20	0.13-0.15	0.09-0.10	0.06-0.07	1.1-23.4
4	0.16-0.19	0.12-0.14	0.08-0.10	0.06-0.07	2.1-23.4
5	0.16-0.18	0.12-0.13	0.08-0.09	0.06-0.06	2.1-23.4
8	0.12-0.14	0.09-0.10	0.06-0.07	0.04-0.05	2.9-23.4
10	0.11-0.12	0.08-0.09	0.05-0.06	0.04-0.04	3.7-36.0
Endospore attachment from field experiments					
0	0.37-0.37	0.27-0.28	0.18-0.19	0.13-0.13	0.1-23.7
1	0.29-0.30	0.22-0.22	0.15-0.15	0.10-0.11	0.2-23.7
2	0.27-0.28	0.20-0.21	0.14-0.14	0.10-0.10	0.3-23.7
3	0.27-0.28	0.20-0.21	0.13-0.14	0.09-0.10	0.3-28.8
4	0.25-0.27	0.19-0.20	0.13-0.14	0.09-0.10	0.7-28.8
5	0.26-0.28	0.19-0.21	0.13-0.14	0.10-0.10	0.8-28.8
8	0.24-0.27	0.18-0.20	0.12-0.14	0.09-0.10	1.3-28.8

^aThe α level used for computation of d is 0.05 (95% confidence) for all situations.

^b T is the tally threshold defined as the maximal number of individuals in a sample unit that may be treated as absent.

estimation from the centrifugal bioassay and medium d values were attained from the incubation bioassay. In most cases, increasing the T values would reduce d . A d value of 0.2 was probably adequate for most research purposes. This required the sample sizes of 25 J2 for the centrifugal bioassay, 25 to 50 J2 for the incubation bioassay, and 50 J2 for field experiments (Table 5.2).

Estimation of the number of endospores attached per J2 from the proportion of J2 with $\leq T$ endospores attached was feasible both theoretically and practically. Using tally thresholds would reduce the time and effort considerably in determining the number of endospores attached per J2. Although this method required a large sample size (25 to 50 J2/sample), it was relatively easy and rapid to determine a J2 with $\leq T$ or $\geq T$ endospores attached, thus making the method less time-consuming. In practice, some J2 had several dozens to more than 100 endospores attached. Attaining an accurate count when a large number of endospores are attached per J2 was both difficult and tedious. The T values in this study were small, and using a tally threshold would avoid counting these large numbers. Thus, by choosing an appropriate T value, especially with high r^2 (Table 5.1) and a desirable sample size, the number of endospores attached per J2 could be determined easily and precisely.

Chapter 6
ULTRASTRUCTURE, MORPHOLOGY, AND SPOROGENESIS OF
PASTEURIA PENETRANS

Introduction

Pasteuria penetrans (Thorne) Sayre & Starr is a widespread mycelial and endospore-forming bacterium that has shown great potential as a biological control agent of plant-pathogenic nematodes (Brown et al., 1985; Chen et al., 1996b; Dickson et al., 1994; Stirling, 1984). It has been reported on 205 nematode species belonging to 96 genera from 51 countries on five continents and on various islands in the Atlantic, Pacific, and Indian oceans (Sayre and Starr, 1988; Sturhan, 1988). *Pasteuria penetrans* isolates have a rather limited host range and exhibit considerable morphological diversity, particularly in size and shape of sporangia and endospores (Sayre and Starr, 1985; Sayre and Starr, 1988).

Four species of *Pasteuria* have been described. *Pasteuria ramosa*, which parasitizes water fleas of the genus *Daphnia* (Sayre et al., 1983), is the type species of the genus. The other three species of *Pasteuria* are parasites of plant-parasitic nematodes: *P. penetrans* on *Meloidogyne* spp. (Sayre and Starr, 1985), *P. thornei* on *Pratylenchus* spp. (Starr and Sayre, 1988), and *P. nishizawae* on cyst nematodes of the genera *Heterodera* and *Globodera* (Sayre et al., 1991a). Two other proposed new species of *Pasteuria* have been isolated from *Heterodera goettingiana* Liebscher in Münster, Germany (Sturhan et al., 1994), and from *Belonolaimus longicaudatus* Rau in Florida (Giblin-Davis et al., 1995). Criteria for the differentiation of these species include size, shape, and structure of endospores and sporangia, developmental morphology of the bacterium, and host specificity (Giblin-Davis et al., 1995; Sayre et al., 1988;

Starr and Sayre, 1989; Sturhan et al., 1994).

Individual isolates of *P. penetrans* exhibit narrow host ranges (Oostendorp et al., 1990; Sayre et al., 1988; Stirling, 1985), but the reasons for the host specificity of *P. penetrans* have not been elucidated (Oostendorp et al., 1990; Stirling, 1985). Several isolates of *P. penetrans* from Florida have varying host specificity (Oostendorp et al., 1990) and are currently being evaluated as biological control agents of root-knot nematodes. The objective of this experiment was to determine whether the ultrastructure of endospores and sporangia or sporogenesis among four Florida isolates of *P. penetrans* are different.

Materials and Methods

Pasteuria penetrans isolates. The designations and origins of the *P. penetrans* isolates were P-20 from *M. arenaria* race 1 (Neal) Chitwood, Levy County; P-100 from *Meloidogyne* sp., Pasco County; P-120 from *Meloidogyne* spp., Alachua County; and B-4 from *Pratylenchus scribneri* Steiner, Seminole County Florida (Oostendorp et al., 1990). Isolate P-20 was propagated on *M. arenaria* race 1, and P-100 and B-4 were propagated on *M. incognita* race 1 (Kofoed & White) Chitwood growing on tomato (*Lycopersicon esculentum* Mill. cv. Rutgers) in a greenhouse. Endospores of each *P. penetrans* isolate were induced to attach separately to newly-hatched second-stage juveniles (J2) of root-knot nematodes using centrifugation (Hewlett and Dickson, 1994). Second-stage juveniles with approximately five endospores attached were inoculated on potted tomato (45-60 days old) at 3,000 J2/pot. Tomato plants were grown in 15-cm-diameter pots and were provided with fertilizer and water as needed. Sporangium-filled females were harvested 55 to 60 days after inoculation. For P-120, sporangium-filled root-knot nematode females were collected from galled tobacco (*Nicotiana tabacum* L.) roots growing in a naturally infested field at the Green Acres Agronomy Farm, University of Florida, Gainesville.

Comparisons of sporangium size. Scanning electron microscopy (SEM) has been used routinely for determining the size of *P. penetrans* sporangia (Mankau, 1975; Sayre and Starr, 1985; Starr and Sayre, 1988). A modified procedure was used to prepare the samples for SEM (Dykstra, 1993). Approximately 20 g (fresh weight) of tomato or tobacco roots containing sporangium-filled females were incubated overnight in 100 ml of a 12.5% cytolase PCL5 solution (Genencor International, Rochester, NY) to loosen root tissue and free root-knot nematode females. Following incubation, the material was decanted onto a 600- μ m-pore sieve nested in a 75- μ m-pore sieve and subjected to a high pressure spray of water to dislodge the females (Hussey, 1971). Sporangium-filled females were hand-picked with forceps with the aid of a stereomicroscope at $\times 20$. They were rinsed three times in deionized water, and individual females were then ruptured with fine forceps and smeared on a 1-cm $\times$ 0.5-cm Whatman No. 1 filter paper (Whatman International, Springfield Mill, Kent, England). Filter paper containing sporangia was immediately transferred to 2% glutaraldehyde in a 0.1 M cacodylate buffer (pH 7.2) and maintained overnight at 4 °C. The fixed samples were rinsed in chilled cacodylate buffer three times, 20 minutes each time, and post-fixed with 2% osmium tetroxide in buffer for 2 hours at room temperature. Samples were rinsed three times, 10 minutes each time, with buffer to remove the excess osmium tetroxide. They were dehydrated through 25%, 50%, 75%, 95%, and 100% ethanol series for 1 hour in each concentration. Exposure to 100% ethanol was repeated three times. Following dehydration, the samples were immersed for 5 minutes in hexamethyldisilazane (Alltech Associates, Deerfield, IL), air-dried overnight in a fume hood, mounted on aluminum stubs, coated with gold, and examined with a Hitachi S-570 scanning electron microscope (Hitachi Instruments, Danbury, CT) operating at 15 kV. The height and diameter of the cup-shaped sporangia were determined from SEM photomicrographs, which were taken using Professional Polaroid 10-cm $\times$ 12.5-cm Instant Film No. 55 (Polaroid,

Cambridge, MA) or with 35-mm negative film.

Effects of nematode host and resistant crops on size of sporangia. Females of *M. incognita* and *M. javanica* infected with *P. penetrans* were collected from a previously identified root-knot nematode suppressive site at the Green Acres Agronomy Farm, University of Florida, Gainesville (Chen et al., 1994). The site was known to be infested with *M. incognita* and *M. javanica*. Two *M. incognita* females and one *M. javanica* female filled with *P. penetrans* were collected from *M. incognita*-resistant tobacco cv. K326 and two *M. javanica* females and one *M. incognita* female filled with *P. penetrans* were collected from the *M. incognita*-susceptible tobacco cv. Hicks. The samples were processed for SEM as described in the previous section. After each female was ruptured and smeared on filter paper, the cuticle of the female was processed for species identification using the perineal pattern (Jepson, 1987).

Attachment of endospores to J2 of *M. arenaria*. Endospores of P-20 were attached to J2 of *M. arenaria* 1 day after eclosion using centrifugation (Hewlett and Dickson, 1994). About 50 J2 with endospores attached were picked immediately with a bristle and rinsed in three changes of deionized water. The protocol previously mentioned for preparation for SEM was used to process the samples. Morphology of endospores attached to J2 was examined with a Hitachi S-570 scanning electron microscope operating at 15 kV.

Morphological comparisons of four isolates of *P. penetrans*. Transmission electron microscopy was used to examine the sporangium morphology of P-20, P-100, P-120, and B-4. Females containing *P. penetrans* were fixed by a microwave fixation method (Login et al., 1992) in Karnovsky's fixative (Karnovsky, 1965) in 0.1 M cacodylate buffer (pH = 7.2) at 50 °C. After fixation, the material was left at room temperature for 1 hour. Nematodes were then ruptured with a surgical knife to facilitate the penetration of solutes and infiltration of the plastic. The ruptured nematodes were embedded in a drop of 1% agarose at 45 °C. Following this, the nematodes were transferred into a second fixative containing 2.5% glutaraldehyde and 5%

dimethyl sulfoxide in 0.1 M cacodylate buffer and kept overnight at 4 °C (Zeikus and Aldrich, 1975).

The specimens were washed twice with the cacodylate buffer for 30 minutes each time, and postfixed with 2% osmium tetroxide at 4 °C overnight (Dykstra, 1993). After rinsing twice in deionized water for 20 minutes each time, the specimens were dehydrated in an ethanol series (25%, 50%, 75%, 95%, and three changes of 100% ethanol) for 1 hour in each concentration. The ethanol was replaced by 100% acetone with two changes for 30 minutes each time. The specimens were infiltrated through a series of Spurr's standard resin in acetone (Spurr, 1969): 30%, 70%, and two changes of 100% resin, overnight each time. Following the infiltration, the specimens were oriented in plastic embedding boats containing fresh 100% resin approximately 3 mm in depth and allowed to polymerize in a 60 °C oven for 48 hours.

Ultrathin sections were cut with a glass or diamond knife on a MT 6000-XL Ultramicrotome (RMC, Tucson, AZ). Gray and silver sections (50-70 nm thick) were mounted on 100-mesh copper grids coated with formvar film and poststained in 1% uranyl acetate for 10 minutes and in saturated aqueous lead citrate for 5 minutes (Dykstra, 1993). The specimens were examined with a JEOL 100 CX transmission electron microscope (JEOL, Tokyo, Japan) at 60 kV.

Terminology. Terminology suggested by Sussman and Halvorson (Sussman and Halvorson, 1966) and recently reviewed by Van Iterson (1984) as applied to *Bacillus* spp. is used to describe the ultrastructure of the developmental stages of *P. penetrans*. The terms used in this paper are: (i) the endospore is the single asexual spore that develops within a sporangium and is enclosed by an exosporium; starting from the innerside to the outside of the endospore, (ii) the plasma membrane is the innermost layer that surrounds the central protoplast and is surrounded by (iii) the cell wall that is usually invisible in transmission electron microphotographs; (iv) the cortex appears as a wide electron-translucent zone that surrounds the cell wall and plasma

membrane; the cortex is followed outside by (v) the inner coat, a much narrower multilaminar band and (vi) the outer coat, a wide electron-dense wall. All of these structures are surrounded by (vii) the exosporium, a delicate membrane that delineates the outermost layer of a typical gram positive bacterial spore. Within the exosporium, *Pasteuria* endospores have additional structures that include (viii) the epicortical layer, which is a discontinuous, electron-dense band around the cortex that varies in appearance from species to species, and (ix) the perisporium, largely consisting of peripheral fibers, which are an essential part of the *Pasteuria* endospore and are probably involved in attachment to the nematode cuticle. The central part of the entire endospore, limited by the outer coat and distinctly differentiated from the perisporium, will be referred to as the “central body” (Sturhan et al., 1994).

Results

The size and shape of the sporangium. Sporangia were cup-shaped and morphologically identical among the isolates (Figs. 6.1, 6.2). Dorsal sides of sporangia were smooth and round. The ventral side was wrinkled (Fig. 6.1). Occasionally, two sporangia were connected. When they were separated, a trace of a “connection ring” was revealed (Figs. 6.1, 6.2). Stem cells were seldom seen in the four isolates (Fig. 6.2).

The average diameter of B-4 sporangia was greater than that of other isolates ($P \leq 0.05$) (Table 6.1). The average height of sporangia of P-120 (1.93 μm) was greater than that of P-100 (1.74 μm) ($P \leq 0.05$). The ranges of sporangium dimensions of the isolates, 2.58 to 3.42 μm in diameter and 1.38 to 2.38 μm in height, however, were similar.

Effects of nematode hosts and resistant crops on the size of sporangia. Diameters and heights of sporangia differed among various combinations of *Meloidogyne* species and resistant

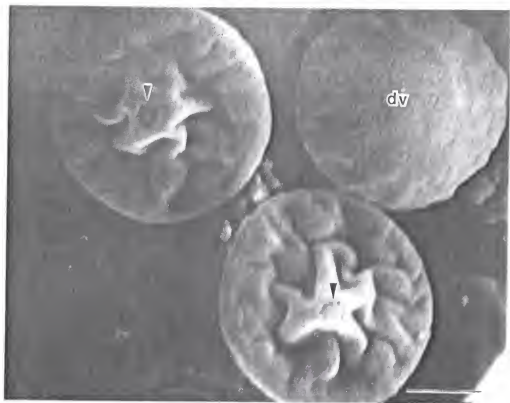


Fig. 6.1. Mature sporangia of *Pasteuria penetrans*. Dorsal view (dv): sporangium is smooth and round. In ventral view, sporangium is wrinkled and has a "connection ring" (arrow head). Scale bar = 1 μm .

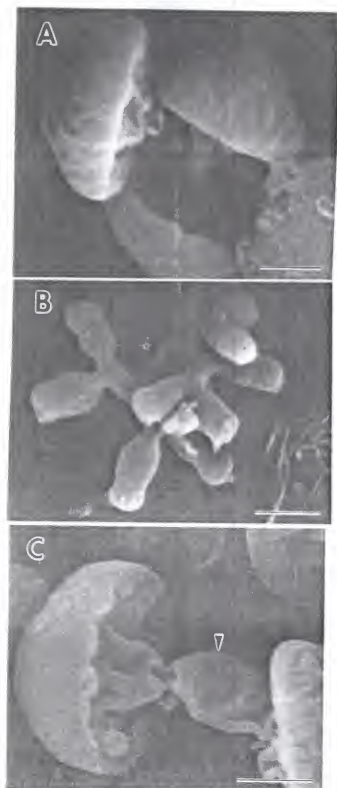


Fig. 6.2. Lateral view of sporangia and early sporogenous stage of *Pasteuria penetrans*. Scale bar = 1 μ m. A) Two sporangia are connected. B) Early sporogenous stage of *Pasteuria penetrans*. The terminal cells increase in size and elongate. C) Lateral view of a sporangium shown connected with a degenerated sporangium or stem cell (arrow head).

Table 6.1. Diameter and height of sporangia from four isolates of *Pasteuria penetrans*.

Isolates ^a	Diameter (μm) ^b			Height (μm)		
	Average	SE	Range	Average	SE	Range
P-20	3.10 b ^c	0.04	2.58-3.34	1.81 ab	0.05	1.38-2.08
P-100	3.07 b	0.03	2.78-3.21	1.74 b	0.04	1.42-2.01
P-120	3.09 b	0.03	2.76-3.30	1.93 a	0.06	1.58-2.38
B-4	3.23 a	0.03	2.84-3.42	1.86 ab	0.04	1.58-2.13

^a Isolates P-20, P-100, P-120, and B-4 originated from *Meloidogyne arenaria* race 1 in Levy County, *Meloidogyne* sp. in Pasco County, *Meloidogyne* spp. in Alachua County, and *Pratylenchus scribneri* in Seminole County, Florida, respectively.

^b Twenty sporangia each were measured from scanning electron photomicrographs at $\times 15,000$. Sporangia of each isolate were processed from a single female.

^c Means in a column followed by different letters are different ($P \leq 0.05$) according to Duncan's multiple-range test.

crops ($P \leq 0.05$) (Table 6.2). Diameters of sporangia obtained from the three *M. incognita* females averaged 3.08 μm , whereas the diameter of sporangium obtained from three *M. javanica* females averaged 3.04 μm . The average sporangium heights were also similar, 1.82 μm for sporangia from *M. incognita* females and 1.92 μm for sporangia from *M. javanica* females. The sporangium diameter averaged 3.06 μm for K326 and 3.07 μm for Hicks. The average sporangium heights were 1.84 μm and 1.90 μm , for Hicks and K326, respectively. Maximum differences in sporangium diameters were less than 0.22 μm ranged across all the treatments, and less than 0.23 μm in the sporangium heights. Thus, the effects of nematode hosts and tobacco cultivars on sporangium size were negligible.

Endospores attached to the second-stage juveniles. Endospores of *P. penetrans* attached along the entire body length of J2 (Fig. 6.3). Following attachment, the sporangial wall and exosporium of most endospores sloughed off (Figs. 6.3-5). This exposed the peripheral fibers and the central body. The endospore and the central body were round (Fig. 6.5). The endospores were $2.81 \pm 0.15 \mu\text{m}$ in diameter. The central body alone measured $1.58 \pm 0.05 \mu\text{m}$ in diameter. Peripheral fibers appeared rough when viewed with SEM (Fig. 6.5A).

Three distinct forms of endospores were observed attached to the cuticle of J2. In one form, which was rare, the endospore surface was covered by the sporangium wall (Fig. 6.3A). In the second form, the sporangium wall sloughed off and the surface was covered by the exosporium (Fig. 6.4B). In the last form, both the sporangium wall and the exosporium sloughed off to reveal the intact peripheral fibers and the central body (Fig. 6.5).

Transmission electron microscopy. Various developmental stages (vegetative, sporogenesis, and mature sporangia) were simultaneously observed in a single female (Fig. 6.6). Ultrastructure and morphology of developmental stages (mycelium and sporogenesis) of the four

Table 6.2. Diameter and height of sporangia of isolate P-120 of *Pasteuria penetrans* sporangia grown on two tobacco cultivars and on two *Meloidogyne* species.

Tobacco cultivar ^a	Host nematode	Diameter (μm) ^b			Height (μm)		
		Average	SE	Range	Average	SE	Range
Hicks	<i>M. incognita</i>	3.19 a ^c	0.04	2.96-3.39	1.79 c	0.06	1.46-2.34
Hicks	<i>M. javanica</i>	3.09 a	0.03	2.75-3.30	1.93 ab	0.06	1.58-2.38
Hicks	<i>M. javanica</i>	2.90 b	0.05	2.39-3.21	1.81 bc	0.04	1.54-2.09
K326	<i>M. incognita</i>	2.97 b	0.04	2.42-3.17	1.82 bc	0.04	1.50-2.08
K326	<i>M. incognita</i>	3.09 a	0.04	2.76-3.34	1.86 bc	0.03	1.63-2.08
K326	<i>M. javanica</i>	3.14 a	0.04	2.84-3.42	2.02 a	0.03	1.76 -2.17

^aHicks is susceptible to *M. incognita* and K326 is resistant.

^bTwenty sporangia each were measured from scanning electron photomicrographs at $\times 15,000$. Sporangia of each treatment were processed from a single female.

^cMeans in a column followed by different letters are different ($P \leq 0.05$) according to Duncan's multiple-range test.

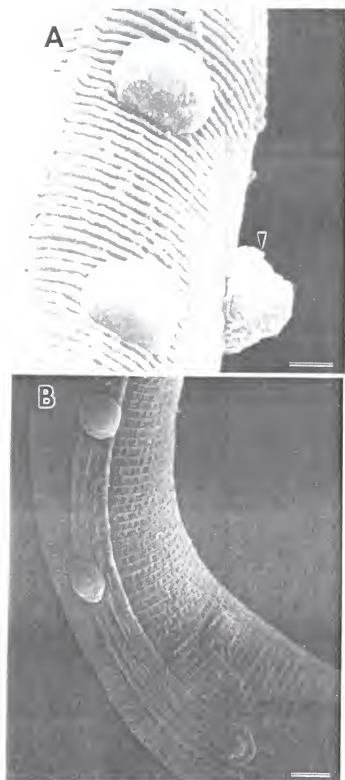


Fig. 6.3. Endospores of *Pasteuria penetrans* attached to the cuticle of a second-stage juvenile (J2) of *Meloidogyne arenaria* race 1. A) Endospores attached to the anterior region of J2. One of the endospores has retained the sporangium wall (arrow head). Scale bar = 1 μ m. B) Endospores attached to the lateral field of J2. Scale bar = 2.5 μ m.

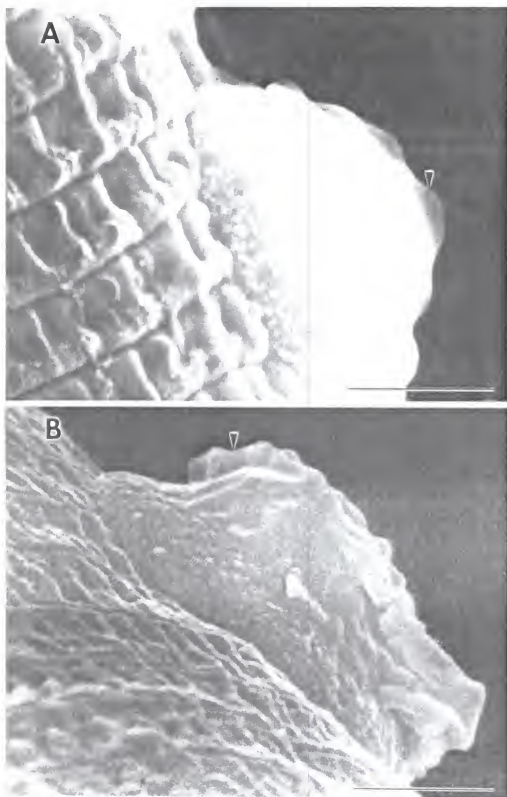


Fig. 6.4. Sporangial wall and exosporium of *Pasteuria penetrans*. Scale bar = 1 μm . A) Sporangial wall and exosporium (arrow head) sloughing off after attachment. B) Endospore with the exosporium (arrow head).



Fig. 6.3. Top view of endospores of *Pasteuria penetrans* attached to the cuticle of second-stage juveniles of *Meloidogyne arenaria* race 1. Scale bar = 1 μ m. A) Both the sporangium wall and the exosporium sloughed off to reveal the peripheral fibers (p) and the central body (c). B) Top view of an endospore. Both the endospore and the central body are round.

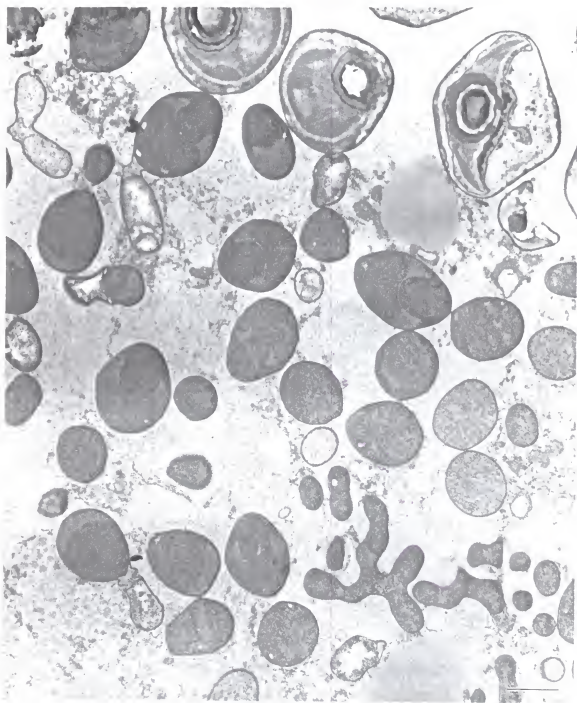


Fig. 6.6. Different developmental stages of *Pasteuria penetrans* within the pseudocoel of a single female. Scale bar = 1 μm .

isolates were similar. Hyphae were septate and bounded by a wall 0.03 μm thick, consisting of an inner and an outer membrane. The inner membrane constricted to form the septations that delineate individual cells (Fig. 6.7). Septations were few in this early rapid growth stage of the microcolony (Fig. 6.7A). The hypha terminal was dichotomously branched. In advanced developmental stages, fragmented thalli separated from the mycelial masses and the terminal cell elongated (Fig. 6.7B). Then, the terminal cells increased in size and became oval (Figs. 6.2B, 6.8A). These cells measured 1.2 to 1.7 μm long and 0.6 to 1.0 μm wide, and were bounded by a wall 0.02 μm thick. A membrane formed about 0.4 μm from the anterior end, and separated the forespore from the parasporium (Fig. 6.8A, B). The forespore was located in the anterior end and was small in size compared with the parasporium. The parasporium continued to increase in size, and gradually engulfed the forespore. Eventually, two-thirds of the forespore was engulfed by the parasporium (Fig. 6.8C). Then, the membrane invagination grew toward the cell pole. When the membrane finally met at the cell pole, the forespore was surrounded by an inner membrane that bounded its own protoplast and an outer membrane that bounded the mother cell's protoplast (Fig. 6.9A). Peripheral fibers formed and attached to the lower part of the forespore (Fig. 6.9A). A cell wall formed covering the inner forespore membrane (Fig. 6.9B). When endospores germinate, these layers develop into the cell wall and the protoplasmic membrane of the germinal tube, respectively. Lamellae were initiated from the base of the forespore and translocated toward the pole, covering the outer forespore membrane. The forespore, 1.1 μm long and 0.7 μm wide, had visible DNA and a boundary wall of 0.01 μm thick (Fig. 6.9). The sporangia of this stage were egg-shaped, 2.0 μm long and 1.5 μm wide, and bounded by a 0.03- μm -thick wall.

The forespore began to form the endospore. The sporangium continued to increase in dimension, with lateral expansion faster than the longitudinal growth. Endospores, at this stage,

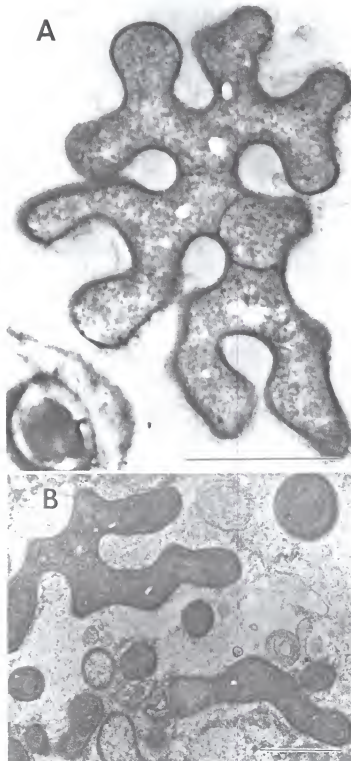


Fig. 6.7. Mycelium of *Pasteuria penetrans*. Scale bar = 1 μm . A) Mycelium of *Pasteuria penetrans* is dichotomously branched (arrow head). Septations are few in this early rapid growth stage of a microcolony. B) Microcolony is fully septate and the terminal cells elongate to give rise to a sporogenous cell. Stage I in the sporogenesis of *Pasteuria penetrans*.

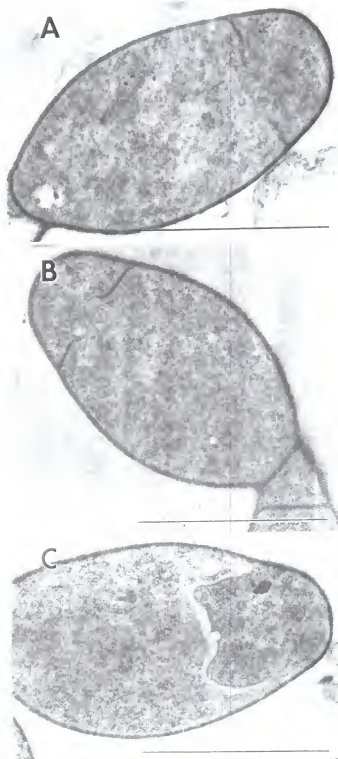


Fig. 6.8. Early sporogenous stages of *Pasteuria penetrans*. Scale bar = 1 μ m. A) The terminal cell of mycelium increase in size and become oval. A membrane forms at $1/3$ distance from the anterior end. Stage II in the sporogenesis of *Pasteuria penetrans*. B) The membrane completely separates the forespore from the parasporium in late stage II. C) In stage III, the parasporium increases in size and engulfs the forespore.

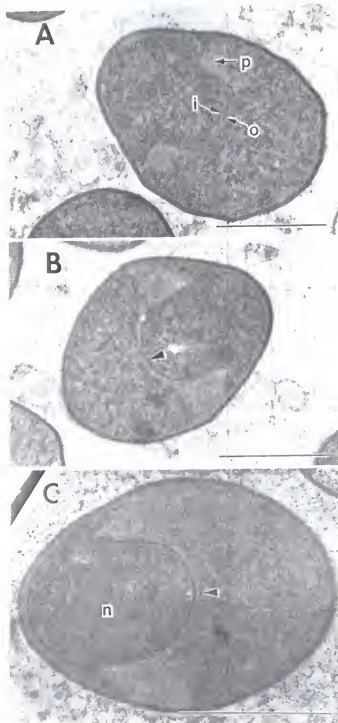


Fig. 6.9. Sporogenous stages of *Pasteuria penetrans*. Scale bar = 1 μm . A) The parasporium completely engulfs the forespore. The forespore is surrounded by an inner membrane (i) that bounds its own protoplast and an outer membrane (o) that bounds the mother cell's protoplast. Peripheral fibers (p) are formed and attach to the lower part of the forespore. B) A cell wall (arrow head) forms to cover the inner forespore membrane in late stage III. C) Lamellae (arrow head), which give rise to the cortex, the inner coat, and the outer coat of the endospore, are initiated from the base of the forespore in early stage IV. The nucleoid (n) contains DNA.

were oval or round and 1.0 μm in diameter. The protoplast of the endospore, except for the light area containing DNA, became electron dense (Fig. 6.10A), indicating dehydration of the endospore protoplast. The cortex, originating underneath the multiple lamellae, was formed. The endospore coat was also formed surrounding the cortex (Figs. 6.10, 6.11). The exosporium was the last membrane formed to cover the endospore (Fig. 6.10B). When the endospore matured, a basal ring was formed surrounding the germinal pore. The epicortical layer was located between the cortex and the inner coat (Fig. 6.12).

In a mature endospore, the cortex and the outer coat measured 0.13 μm and 0.15 μm thick, respectively. Unlike the cortex, the outer coat tapered toward the basal side of the endospore, measuring only 0.03 μm thick in the germinal pore. The germinal pore was 0.4 μm in diameter and surrounded by a basal ring that was texturally similar to the outer coat (Fig. 6.13). The central body of the endospore was ellipsoidal, $1.4 \times 0.9 \mu\text{m}$ in dimension, with the long axis lying horizontally. The epicortical layer was discontinuous, only visible on the lateral sides of the endospore. A line drawing of sporogenesis in *P. penetrans* is shown in Fig. 6.14.

Discussion

Ultrastructure, morphology, and morphometrics of sporangia were similar among the Florida isolates. Sporangia of the four isolates were 2.58 to 3.42 μm in diameter and 1.38 to 2.38 μm in height, which were smaller than those reported previously (3.0 to 4.0 μm in diameter and 2.26 to 2.6 μm in height) (Starr and Sayre, 1988). These differences could be caused by the different methods used to prepare samples for electron microscopy (Starr and Sayre, 1988). Morphology of mature sporangia of four Florida isolates was the same as that reported for *P. penetrans* (Mankau, 1975; Sayre and Starr, 1985; Starr and Sayre, 1988), but different from that

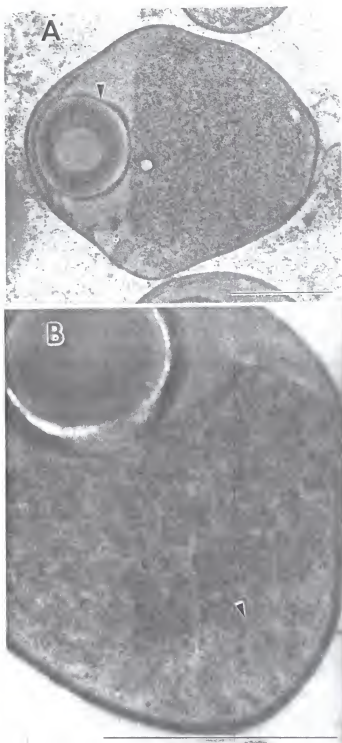


Fig. 6.10. Late sporogenous stages of *Pasteuria penetrans*. Scale bar = 1 μm . A, The outer coat (arrow head) is formed surrounding the cortex. B, Exosporium (arrow head) is formed.

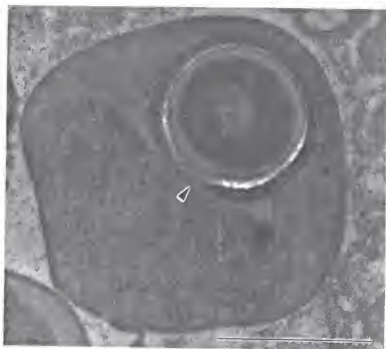


Fig. 6.11. Endospore of *Pasteuria penetrans* in the late sporogenous stage. Arrow head indicating position of the germinal pore of the endospore. Scale bar = 1 μ m.



Fig. 6.12. Maturation of *Pasteuria penetrans* endospores (Stage VII). Epicortical layer (e) is a discontinuous membrane located inside the inner coat (ic) and outer coat (oc). Basal ring (br) is formed surrounding the germinal pore. The peripheral fibers (pf) are an essential part of the *P. penetrans* endospore and allow the endospore to attach to the nematode cuticle. Scale bar = 1 μm .

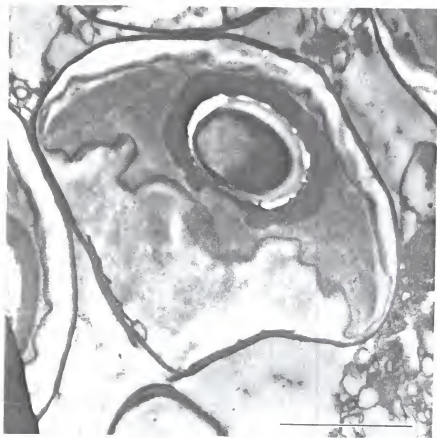


Fig. 6.13. A mature endospore of *Pasteuria penetrans*. Scale bar = 1 μm .

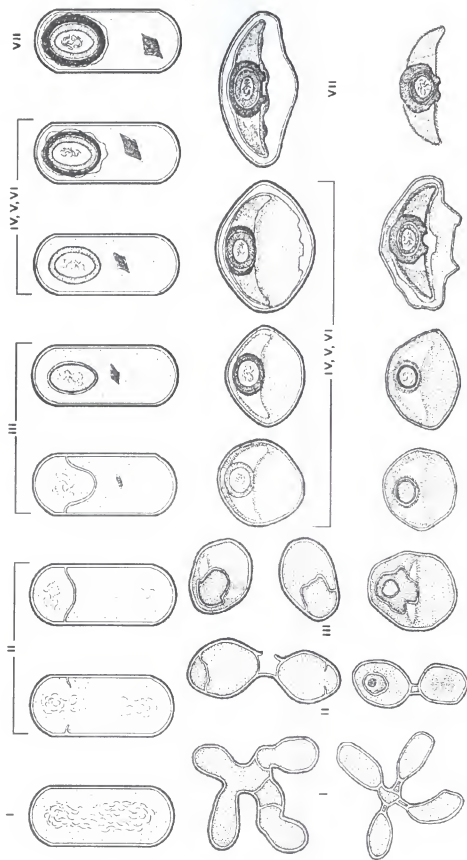


Fig. 6.14. Line drawing of sporogenesis in *Pasteuria penetrans* (middle row), compared with *Bacillus thuringiensis* (upper row, redrawn from van Iterson [1984]) and previously reported sporogenesis of *P. penetrans* (lower row, redrawn from Sayre and Starr [1985]).

of *P. ramosa* (Sayre et al., 1983), *P. thornei* (Starr and Sayre, 1988), *P. nishizawae* (Sayre et al., 1991a), and other *Pasteuria* spp. (Giblin-Davis et al., 1995; Sturhan et al., 1994).

The Florida isolates exhibited the same ultrastructure, morphology, and sporogenesis, but differed in their host specificity (Oostendorp et al., 1990). Ciancio (1995) reported that morphology and morphometrics of endospores were related to host range across nematode genera. Endospores from different nematode genera usually exhibited differences in ultrastructure, morphology, and morphometrics (Ciancio, 1995; Giblin-Davis et al., 1995; Mankau, 1975; Sayre and Starr, 1985; Sayre et al., 1991a; Starr and Sayre, 1988; Sturhan et al., 1994). The Florida isolates used in the present study originated from *Meloidogyne* spp. and *Pratylenchus scribneri*, but all isolates were reared on *Meloidogyne* spp. Stirling speculated that host specificity of *P. penetrans* might be a response to nematode populations rather than to nematode species (Stirling, 1985). All of the isolates reported (Oostendorp et al., 1990; Stirling, 1985) attach to more than one *Meloidogyne* species. A recent study showed that *P. penetrans* can produce heterogeneous endospores (Davies et al., 1994). These heterogeneous subpopulations of endospores show specificity to different nematode populations. It also has been reported that endospores usually attach more readily to the nematode species on which *P. penetrans* is originally obtained or cultured than to other species (Davies et al., 1994; Oostendorp et al., 1990). Thus, the host specificity of *P. penetrans* may result from the adaptation of the bacterium to its host nematodes. Such adaptations probably were not followed by changes in the ultrastructure, morphology, and morphometrics of endospores.

The differences in sporangium size of the four Florida isolates were slight but statistically different ($P \leq 0.05$). Bird et al. (1990) also found different sizes of sporangia among different isolates. Their conclusion that these differences are too small to reliably differentiate various isolates is supported by the present study.

Two forms of endospores have been observed attached to the second-stage juveniles (J2) of *Meloidogyne* spp.: endospores covered with an exosporium and bare endospores (Sayre and Starr, 1985; 1989; Starr and Sayre, 1988). The exosporium in the former, however, resembles the sporangium wall in our observation. The sporangium wall is much thicker than the exosporium. The appearances of exosporium and sporangium wall are different in the scanning electron photomicrographs. The wrinkled appearance of the sporangial wall, which probably led Sayre and Starr (Sayre and Starr, 1985; 1989; Starr and Sayre, 1988) to consider it as the exosporium, may result from the attachment of the endospore to the juvenile cuticle. Mature endospores usually retain their exosporium and sporangial wall. When using centrifugation for attaching endospores to J2 (Hewlett and Dickson, 1994), we found that most of the attached endospores were without sporangial walls. Some unknown mechanisms must be involved in the rapid sloughing-off of the sporangial wall and exosporium. One possible explanation is that when the endospore descends to the nematode surface, the centrifugal force ruptures the sporangial wall and allows the endospore to contact the surface of the juvenile's cuticle. Following this initial contact, the peripheral fibers conform with the cuticle to establish a firm attachment. This process may also result in the sloughing-off of the sporangial wall and exosporium because these layers are not connected to the endospore. Endospores that retain the sporangial remnants probably are the intermediate forms, or the uncompleted forms, of the endospore attachment to J2.

Mesosomes were observed in *P. penetrans* (Imbriani and Mankau, 1977; Mankau, 1975; Sayre, 1993; Sayre and Starr, 1985; 1988; 1989; Sayre et al., 1983; Starr and Sayre, 1988), *P. ramosa* (Sayre, 1993; Sayre and Starr, 1988; 1989; Sayre et al., 1983; Starr and Sayre, 1988), and *P. nishizawae* (Sayre and Starr, 1988; Sayre et al., 1991a), but not in *P. thornei* (Sayre and Starr, 1989; Starr and Sayre, 1988) and other *Pasteuria* species (Giblin-Davis et al., 1995; Sayre et al., 1991b). Mesosomes were not observed in the four Florida isolates. Mesosomes also have

been observed in other bacteria (Van Iterson, 1984). Based on recent evidence from freeze-fracture preparations, however, these internal membranous structures probably are fixation artifacts (Dubochet et al., 1983; Ebersold et al., 1981; Nanninga, 1971). Moreover, a recent study has proved that suboptimum fixation conditions could create the artifact (Aldrich et al., 1987). These conditions include any one of the following fixatives: phosphate-buffered 2.5% glutaraldehyde, 0.1% osmium tetroxide, and 0.5% glutaraldehyde plus 2.5% formaldehyde (Aldrich et al., 1987). Mesosomes reported in *Pasteuria* spp. were always associated with material fixed with 2% or 3% glutaraldehyde in phosphate buffers (Imbriani and Mankau, 1977; Mankau, 1975; Sayre, 1993; Sayre and Starr, 1985; 1988; 1989; Starr and Sayre, 1988; Sayre et al., 1983; 1991b). Materials freeze-fractured, freeze-substituted, or fixed in cacodylate-buffered 2.5% glutaraldehyde lacked mesosomes (Aldrich et al., 1987).

Transmission electron micrographs in earlier studies of *P. penetrans* only showed a few stages of sporogenesis (Mankau, 1975; Sayre, 1993; Sayre and Starr, 1985; 1989; Sayre et al., 1983; 1988; Starr and Sayre, 1988). Furthermore, some previous transmission electron micrographs, such as an opaque area in the forespore (Fig.5 in Sayre and Starr [1985] and Fig. 33.25 in Sayre and Starr [1989]), were difficult to interpret. Accordingly, the line drawings reported in the earlier studies represented only a portion of the sporogenesis in *P. penetrans* (Sayre and Starr, 1985). The results of the present study provide a more complete scenario of sporogenesis than those provided by other studies (Mankau, 1975; Sayre, 1993; Sayre and Starr, 1985; 1989; Sayre et al., 1983; 1988; Starr and Sayre, 1988).

The line drawings of the transmission electron micrographs reveal that sporogenesis in *P. penetrans* is similar to that of other well-studied gram positive bacteria. Here, *Bacillus thuringiensis* was chosen for comparison. The sporogenesis in *B. thuringiensis* has been classified into seven morphological stages (Van Iterson, 1984). Stage I, defined as condensation of the replicated chromosome to form an axial filament, is not observed in *P. penetrans*. Stages

II to VII are similar in *P. penetrans* and *B. thuringiensis* and involve formation of the forespore septum (Stage II); engulfment of the forespore (Stage III); formation of the cortex, the coat, and the exosporium (Stages IV to VI); and sporangium maturation (Stage VII). Perisporium, which is termed peripheral fibers in *P. penetrans* and parasporal crystals in *B. thuringiensis*, is formed after engulfment of the forespores at stage III in both organisms.

Although *P. penetrans* and *B. thuringiensis* exhibit similar sporogeneses, they are not necessarily close relatives. Sporogenesis and ultrastructure of bacterium endospores are highly conserved (Priest, 1993). Sporogenesis of bacteria probably evolved only once (Priest, 1993). Moreover, *P. penetrans* and *B. thuringiensis* differ in several fundamental ways. Most importantly, *P. penetrans* differs from *B. thuringiensis* in that *P. penetrans* penetrates its host with a germ tube and then forms thalli in the host body cavity. Since artificial cultivation of *Pasteuria* spp. is presently unsuccessful, their phylogenetic relationship to the other gram positive bacteria remains unknown (Berkeley and Ali, 1994).

Chapter 7 SUMMARY

Plant-parasitic nematodes cause a \$6 billion monetary loss to United States agriculture each year (Sasser and Freckman, 1987). Currently, the management of plant-parasitic nematodes relies largely on a combination of crop rotation and nematicides. Environmental hazards of nematicides, however, have led to great controversy about their continued use. Biological control of nematodes offers an alternative or supplemental management tactic to chemical or cultural control of nematode pathogens. The biology of *Pasteuria penetrans* and its potential for management of *Meloidogyne arenaria* (Neal) Chitwood race 1 on peanut (*Arachis hypogaea* L.) were investigated in laboratory, greenhouse, and field experiments.

Six methods for quantification of the endospore concentrations of *P. penetrans* from tomato roots were compared. Mortar disruption and machine disruption methods gave the highest estimations, 83.7 and 79.0 million endospores per gram of root material, respectively. These methods were significantly superior to methods using incubation bioassay (47.7 million), enzymatic disruption (32.1 million), and enzymatic disruption + flotation (25.8 million). A centrifugation bioassay method gave the lowest estimation of 12.7 million.

Pasteuria penetrans was tested over a 2-year period in a field microplot experiment. Endospores were inoculated into the top 20 cm of microplots in the first year only at 0, 1,000, 3,000, 10,000, and 100,000 endospores/g of soil, respectively. The microplots were previously infested with *M. arenaria* race 1. In the first year, root gall indices and pod galls per microplot were significantly reduced by 60% and 95% at 100,000 endospores/g of soil, and 20% and 65% at 10,000 endospores/g of soil, respectively. *Pasteuria penetrans* significantly reduced the densities of second-stage juveniles (J2) of *M. arenaria* that overwintered. In the second year, root and pod

gall indices, respectively, were significantly reduced by 81% and 90% at 100,000 endospores/g of soil, and by 61% and 82% at 10,000 endospores/g of soil. Pod yields were significantly increased by 94% at 100,000 and by 57% at 10,000 endospores/g of soil, respectively. The minimum number of endospores required for significantly suppressing *M. arenaria* race 1 on peanut was 10,000 endospores/g of soil.

Quantitative suppression of *M. arenaria* race 1 on peanut by *P. penetrans* was evaluated using a 6×6 factorial experiment in field microplots over 2 years. The main factors were six inoculum levels of J2 of *M. arenaria* race 1 (0, 40, 200, 1,000, 5,000, and 25,000 J2/microplot, except that the highest level was 20,000 J2/microplot in 1995) and six infestation levels of *P. penetrans* as percentages of J2 with endospores attached (0, 20, 40, 60, 80, and 100%). The results were similar in 1994 and 1995. Numbers of eggs per root system, J2 per 100 cm³ of soil at harvest, root galls, and pod galls increased with increasing nematode inoculum levels and decreased with increasing *P. penetrans* infestation levels ($P \leq 0.05$). There were no interactions between the inoculum levels of J2 and the infestation levels of *P. penetrans* ($P > 0.05$). When the infestation level was increased by 10%, the number of eggs per root system, root galls, pod galls, and J2 per 100 cm³ of soil decreased by 8.6%, 7.8%, 8.4%, and 8.8%, respectively ($P \leq 0.05$).

The estimation of attachment of *P. penetrans* endospore to J2 of *Meloidogyne* spp. was determined using tally thresholds. Three data sets with 70, 33, and 111 estimates of mean endospores attached per J2 (m) originated from centrifugal bioassay, incubation bioassay, and field experiments, respectively. Taylor's power law analyses indicated that the means of endospores per J2 had a contagious spatial distribution. Therefore, empirical relationships between m and proportions of J2 with $\leq T$ (tally threshold) endospores attached (P_T) was developed using parameters from the linear regression of $\ln(m)$ on P_T ($0 < P_T < 1$); $\ln(m) = a + b P_T$, and T was set to 0, 1, 2, 3, 4, 5, 8, and 10 endospores/J2. The results indicated that the variances of linear equations tended to decrease with increasing T values for all three data sets. For all T values, $\ln(m)$ regressed well to P_T ($P \leq 0.01$), except at $T = 10$ for data from field experiments in which $\ln(m)$ did not regress to P_T ($P > 0.05$). Values of $T = 0, 1, 8$, and 10

endospores/juvenile for the centrifugal bioassay and incubation bioassay, and 0, 1, 2, and 3 endospores/juvenile for field experiments were associated with an $r^2 \geq 0.8$. These T values may be used in estimating m from P_T to reduce variability and achieve an acceptable error level. Based on these T values, sample sizes of 25 J2 for the centrifugal bioassay, 25 to 50 J2 for the incubation bioassay, and 50 J2 for field experiments were required to achieve a half-width confidence interval of ≤ 0.2 ($\alpha = 0.05$).

Scanning electron microscopy and transmission electron microscopy were used to study the ultrastructure, morphology, and sporogenesis of four Florida isolates of *P. penetrans*. The effects of different *Meloidogyne* spp. and tobacco cultivars (*Nicotiana tabacum* L.) on the sporangium size and morphology of endospores attached to the cuticle of J2 also were investigated. Sporangia of four Florida isolates were morphologically identical and similar in their dimensions, ranging from 2.39 to 3.42 μm in diameter and 1.38 to 2.38 μm in height. Various *Meloidogyne* spp. or different tobacco cultivars had no effect on sporangium size. Sporogenesis of *P. penetrans* was similar to that of other gram positive bacteria and generally followed the seven-stage scheme reported for *Bacillus thuringiensis*.

The present studies provided important information on the biology and efficacy of *P. penetrans* as a biological control agent of *M. arenaria* race 1 on peanut. Further studies, however, are needed to determine the amplification and survival of endospores in nature. The taxonomy of the genus *Pasteuria* needs to be clarified to help solve the complex relationship of various isolates to nematode host populations. Efforts should be directed toward the artificial cultivation of *P. penetrans* to provide mass production of endospores for managing the root-knot nematodes.

APPENDIX
EFFECT OF AMMONIUM NITRATE AND TIME OF HARVEST ON
MASS PRODUCTION OF *PASTEURIA PENETRANS*

Introduction

Plant-parasitic nematodes cause a \$6 billion monetary loss to United States agriculture each year (Sasser and Freckman, 1987). Currently, the management of plant-parasitic nematodes relies largely on a combination of crop rotation and nematicides. Economic considerations and potential environmental hazards of nematicides, however, have led to great controversy about their continued use. Biological control of nematodes offers an alternative or supplemental management tactic to chemical or cultural control of nematode pathogens. One nematode antagonist in particular, *Pasteuria penetrans*, has shown great potential for biological control of root-knot nematodes (Dickson et al., 1994; Mankau and Prasad, 1977; Sayre and Starr, 1985; Stirling, 1984; 1991). *Pasteuria penetrans* is an obligate, mycelial, endospore-forming bacterial parasite of *Meloidogyne* spp. Endospores of *P. penetrans* attach to the cuticle of the second-stage juvenile (J2) of *Meloidogyne* spp. in soil. Infection occurs after the J2 enters a plant root, where parasitized nematodes mostly develop into females, but are incapable of reproduction (Bird, 1986). The females, and sometimes males (Hatz and Dickson, 1992), become filled with endospores, which are eventually released into the soil upon host disintegration.

Pasteuria penetrans has been observed in nematode-suppressive soils and successfully tested against root-knot nematodes in greenhouse and field microplot experiments (Brown et al., 1985; Chen et al., 1996b; 1997a; Dickson et al., 1994; Stirling, 1984). Experiments have demonstrated that the suppressiveness of soil to nematodes can be increased following

continuously planting of susceptible hosts of root-knot nematodes (Oostendorp et al., 1991a). The number of endospores of *P. penetrans* attached per second-stage juvenile (J2) increased correspondingly with the increasing soil suppressiveness. Furthermore, using various techniques to discriminatively eliminate nematodes, fungi, and other bacterial antagonists, *P. penetrans* has been successfully documented as the leading suppressive agent in a nematode-suppressive soil (Weibelzahl-Fulton et al., 1996). *Pasteuria penetrans* also has suppressed root-knot nematodes following application of endospores in field microplots. Reduction of root galls and pod galls and increases in peanut yield have been correlated with increasing levels of *P. penetrans* (Chen et al., 1996b). These effects have been verified in a six $\times$ six factorial experiment, in which root galls, pod galls, number of eggs per root system, and J2 per 100 cm³ of soil increased with increasing nematode inoculum levels and decreased with increasing *P. penetrans* infestation levels (Chen et al., 1996b). The capability of *P. penetrans* in suppressing root-knot nematodes underscores its potential as a successful bio-nematicide. Chen et al. (1996b), as an example, successfully demonstrated that applications of 10,000 endospores/g of soil effectively suppressed root-knot nematodes in peanut microplots.

The major obstacle for the practical application of *P. penetrans* to soil infested with root-knot nematodes is the lack of a sufficient production of endospores. Currently, the parasite must be produced in *Meloidogyne* spp., which, in turn, must be reared on susceptible plants (Stirling and Wachtel, 1980) or in nematode-excised root systems (Verdejo and Jaffee, 1988). The mass production system developed by Stirling and Wachtel (1980) is widely used to produce inoculum for experimental purposes, but several factors affect the mass production of *P. penetrans*. Sharma and Stirling (1991) suggested that nematode inoculum density, plant species, and time of harvest are the main factors influencing endospore production. The optimum conditions suggested are 5,000 J2/pot of inoculated tomato plants and harvest at 900 degree-days (Sharma and Stirling, 1991). Davies et al. (1991) observed that a watering regime using less water favors

the development of *P. penetrans*. The optimum temperature for the development of *P. penetrans* was determined to be 35 °C, and the production of endospores increased with increasing temperature at the range of 20 °C to 35 °C (Hatz and Dickson, 1992). Nitrogen fertilizer is an important factor in greenhouse culture practice. The effect of nitrogen on development of *P. penetrans* remains unknown. In this study, the effects of varying levels of ammonium nitrate and various harvest times on the mass production of *P. penetrans* using a greenhouse pot culture technique were investigated.

Materials and Methods

Nematode isolate: An isolate of *Meloidogyne arenaria* (Neal) Chitwood race 1 from Levy County, Florida, was used. The isolate was cultured on tomato (*Lycopersicon esculentum* Mill. cv. Rutgers) in a steam-pasteurized potting soil. Eggs of *M. arenaria* were extracted from roots in 0.5% sodium hypochlorite for 30 seconds (Hussey and Barker, 1973) and caught on a sieve with 25- μ m-pore openings, rinsed, and placed on Baermann funnels. The roots and plant debris were caught and separated from the eggs by a sieve with 75- μ m-pore openings nested on a sieve with 25- μ m-pore openings. Second-stage juveniles that hatched on the first day were discarded to avoid using nematodes that may have acquired endospores in the potting soil. The juveniles were collected by decanting the water suspension from Baermann funnels onto a sieve with 25- μ m-pore openings, rinsing the juveniles, and transferring them into a beaker. The number of J2 in the suspension was determined by counting five, 1-ml aliquots under a compound microscope.

Pasteuria penetrans isolate: Isolate P-20 of *P. penetrans*, derived from *M. arenaria* race 1 from a peanut field in Levy County, Florida, was propagated on *M. arenaria* race 1 growing on tomato plants in a greenhouse. Endospore-encumbered J2 (up to 5 days old) were obtained by a centrifugal technique (Hewlett and Dickson, 1993). Potted tomato plants (45 to 60 days old)

were inoculated three times with the endospore-encumbered J2 (1,000-1,500 J2/pot/time) at intervals of 1 week. The tomato plants were grown in 15-cm-diameter pots. Averaged over all inoculations, $96 \pm 5\%$ of the J2 had at least one endospore attached, with an average of 4.9 ± 2.0 endospores/J2 among those J2 with endospores. These estimates were made by counting 20 J2/aliquot. Insecticide applications and water were provided as needed. Root systems containing endospore-filled females were harvested 45 to 60 days after the last inoculation, rinsed, and air-dried. The females containing endospores were obtained using an enzymatic disruption method and ground in deionized water using a glass tissue grinder (Chen et al., 1996a). Endospore concentrations of the suspensions were determined by counting five, 0.1-ml aliquots using a hemocytometer (Fisher Scientific No. 02-671-10) at $\times 450$.

Fertilizer test: The experiment was conducted in a shade house at the University of Florida, Gainesville. Fifty-day-old tomato seedlings were transplanted into 15-cm-diameter pots. After 2 days, the potted tomato plants were fertilized with 0.4 g of a 20-20-20 (N-P-K) mixture/pot. Tomato plants were acclimated in the shade house for 18 days before the treatments began. After acclimation, on 1 August 1994, tomato plants were inoculated with endospore-encumbered J2 at 1,500 J2/pot. The incidence of endospore attachment was 3.4 ± 2.1 endospores/J2 and each J2 had at least one endospore attached. After 1 week, tomato plants were treated with various levels of ammonium nitrate.

The experiment had five treatments with six replicates for each treatment. Ammonium nitrate (35% N) was used as the nitrogen source and was applied weekly in 150 ml of water at 0, 0.2, 0.4, 0.8, or 1.6 g/pot. The pots were arranged in a random, complete block design. Fertilizer application was stopped 1 week before harvest; hence, a total of five treatments with a total amount of 0, 1.0, 2.0, 4.0, and 8.0 g of ammonium nitrate per pot applied during the experiment period were applied. Tomato root systems were harvested 51 days after inoculation and stored at 4 °C until examined. The root material was then incubated in 50 ml of a 12.5% PCL cytolase

solution (Genencor International, Rochester, NY) for 2 days, the material was then decanted onto a sieve with 600- μ m-pore openings nested in a sieve with 75- μ m-pore openings and subjected to a high-pressure spray of water to dislodge the females (Hussey, 1971). The females were hand-picked with a pipette (Fisher Scientific No. 13-678-20A, Atlanta, GA) and enumerated with the aid of a microscope at $\times 20$. They were ground in deionized water using a glass tissue grinder. Concentrations of endospores in the suspensions were determined by counting four, 0.1- μ l aliquots using a hemocytometer (Fisher Scientific No. 02-671-10, Atlanta, GA) at $\times 450$.

Time of harvest test: The experiment was conducted in the same shade house and at the same time as the above fertilizer test. The preparation of pots and the inoculation of endospore-encumbered J2 were the same as in the fertilizer test. Tomato plants were fertilized weekly with 1.4 g of a 20-20-20 (N-P-K) mixture/pot beginning 1 week after inoculation of endospore-encumbered J2.

The experiment had five treatments (harvest times) with six replicates per treatment. The pots were arranged in a random complete block design. Tomato root systems were harvested at 37, 44, 51, 58, and 65 days after inoculation and stored at 4 °C. The same methods as in the above fertilizer test were used to determine the number of females and the number of endospores per root system.

Aerial temperature in the shade house was recorded using a maximum-minimum thermometer (Fisher Scientific No. 15-093, Atlanta, GA). Phytotoxicity was observed in the treatment with 1.6 g ammonium nitrate/pot in the fertilizer test, and some second generation females appeared at the harvest time of 65 days; consequently, these two treatments were excluded from the analyses of results. Before analysis, data were transformed to their natural logarithms. Data presented in the text were back-transformed. Regression analysis was used, and an equation was provided if the correlation was significant at $P \leq 0.05$. Duncan's multiple-range

test was used to compare differences among treatment means when the regression was not significant ($P > 0.05$).

Results and Discussions

Ammonium nitrate had adverse effects on the production of *P. penetrans* ($P \leq 0.05$) (Fig. A1). A quadratic relationship was established between the number of females per root system and the ammonium nitrate levels (Fig. A1A). The number of endospores per root system and the number of endospores per female were negatively correlated with the ammonium nitrate levels (Fig. A1B,C). According to the linear regressions, the number of endospores per root system and the number of endospores per female decreased 1.1 million and 0.013 million, respectively, per 0.1 g ammonium nitrate increment.

Supplemental nitrogen is known to suppress plant-parasitic nematodes (Khan et al., 1986; Riispere, 1990; Rodriguez-Kabana, 1986; Talavera et al., 1984; Verma and Gupta, 1987). The suppression mechanisms have not been elucidated. The suppression probably results from either the direct effects of nitrogen on nematodes (Talavera et al., 1984; Verma and Gupta, 1987), the indirect mediation of the physiological state of the feeding site, or an impact on the entire host plant (Riispere, 1990). Usually, the best conditions for development of nematodes occur when the host is under moderate stress (Riispere, 1990). The results of the present study indicate that such suboptimal conditions may also favor the development of *P. penetrans*. Davies et al. (1991) observed that the development of *P. penetrans* in infected females was slower when soil moisture was maintained at field capacity. Since *P. penetrans* is an obligate endoparasite of root-knot nematodes, coevolution of the parasite and the host may have resulted in similar environmental requirements for development.

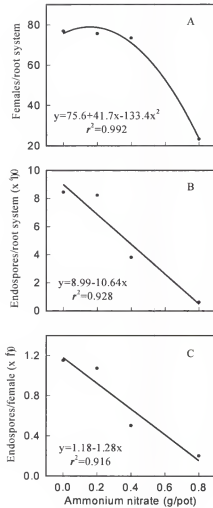


Fig. A1. Effect of ammonium nitrate on *Pasteuria penetrans* grown in *Meloidogyne arenaria* race 1, which in turn was reared on tomato (*Lycopersicon esculentum* Mill. cv. Rutgers) in a shade house under varying temperature conditions. All of the regressions were significant at $P \leq 0.05$. A) A quadratic relationship between the number of females per root system and the ammonium nitrate levels. B) Linear regression of number of endospores per root system to ammonium nitrate levels. C) Linear regression of number of endospores per female to ammonium nitrate levels.

The highest level of ammonium nitrate was remarkably detrimental to the development of root-knot nematode females (Fig. A1A). The number of females per root system at 0.8 g of ammonium nitrate/pot was less than those at other ammonium nitrate levels ($P \leq 0.05$). This may have resulted from the direct injury of nematodes (Talavera et al., 1984). Numbers of females per root system at 0.2 to 0.4 g ammonium nitrate/pot were similar to that in the control (Fig. A1A). Endospore production, however, decreased constantly with increasing ammonium nitrate levels (Fig. A1B, C). This may suggest that development of *P. penetrans* is hampered by ammonium nitrate. Thus, the reduction in the number of endospores per root system resulted from two factors: a reduction in the number of females per root system and a reduction of endospore produced per female. The mode of action of ammonium nitrate on development of *P. penetrans* is unknown, but it probably results from the modification of the physiological state of the nematode hosts. Verma and Gupta (1987) reported that nitrogen was capable of inhibiting the reproduction of root-knot nematodes.

Numbers of endospores produced per root system and per female were comparable to those observed in other studies (Chen et al., 1994; Chen et al., 1996a; Hatz and Dickson, 1992; Verdejo and Jaffee, 1988), but lower than numbers produced in other tests (Davies et al., 1988; Mankau and Prasad, 1977; Stirling, 1981; Stirling and Wachtel, 1980). The number of endospores per female varied from 0.9 million (Davies et al., 1988), 1.4 to 2.4 million (Stirling, 1981), 2 million (Mankau and Prasad, 1977) to 0.1 to 0.2 million (Chen et al., 1994) and 0.08 to 0.15 million (Verdejo and Jaffee, 1988). The ratios of numbers of mature females to numbers of J2 inoculated were reported to be 0.004 to 0.035 (Davies et al., 1991), 0.21 to 0.34 (Stirling, 1984), and 0.13 to 0.21 (Davies et al., 1988) for J2 infested with *P. penetrans* endospores and 0.15 to 0.16 (Davies et al., 1991), 0.57 (Stirling, 1984), and 0.21 to 0.66 (Davies et al., 1988) for noninoculated J2. Apparently, fewer females developed when J2 were encumbered with *P. penetrans* endospores. The ratio of numbers of mature females to numbers of inoculated J2 in

this study was 0.016 to 0.088, which is consistent with the reported ranges. The variations among the different reports on number of females per root system and number of endospores per female could result from the differences in *P. penetrans* isolates, nematode species, plant hosts, and cultural conditions.

Numbers of females recovered from root systems were 41.3, 132.7, 30.4, and 28.2 for the harvest times of 37, 44, 51, and 58 days after inoculation, respectively (Fig. A2A). The number of females per root system at 44 days was greater than that at any other harvest time ($P \leq 0.05$), but it was considered to result from experimental variation. Generally speaking, the number of females per root system should decrease with increasing harvest time, because the natural mortality of females was inevitable. Numbers of endospores per root system were 0.08, 2.74, 0.78, and 2.16 million for the harvest times of 37, 44, 51, and 58 days, respectively (Fig. A2B). The number of endospores per root system at 37 days was lower than that at other harvest times ($P \leq 0.05$), but there were no differences in endospore production at harvest times of 44, 51, and 58 days ($P > 0.05$). The number of endospores per female correlated with the harvest time ($P \leq 0.05$) (Fig. A2C). Endospores per female increased 0.03 million per 10-day increment of harvest time from 37 to 58 days.

During the experiment, the daily high temperature, low temperature, and mean temperature recorded in the shade house were 34.5 ± 3.5 °C, 23.1 ± 1.4 °C, and 28.8 ± 2.2 °C, respectively. The accumulative degree days above 10 °C were 727.8, 858.8, 984.3, and 1,090.3 for the harvest times of 37, 44, 51, and 58 days, respectively. The base threshold temperature of 10 °C, which was originally reported for *M. arenaria* (Ferris et al., 1978), has been used widely to calculate the accumulative degree days for the development of *P. penetrans* (Bird, 1986; Serracin-Ulate, 1996; Sharma and Stirling, 1991; Stirling, 1981). Recently, the base threshold

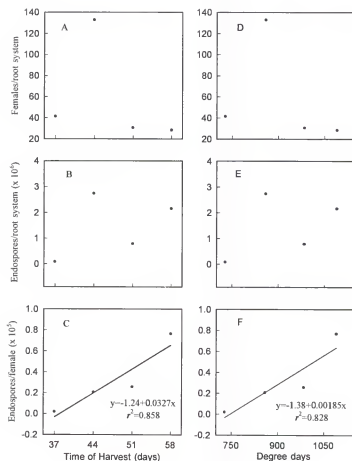


Fig. A2. Effect of harvest time on *Pasteuria penetrans* grown on *Meloidogyne arenaria* race 1, which in turn was reared on tomato (*Lycopersicon esculentum* Mill. cv. Rutgers) in a shade house under varying temperature conditions. The same letter in each graph indicated no difference according to Duncan's multiple-range test ($P > 0.05$). The developmental threshold used for calculating degree days was 17 °C. A) Correlation of number of females per root system to harvest time was not significant ($P > 0.05$). B) Correlation of number of endospores per root system to harvest time was not significant ($P > 0.05$). C) Linear regression of number of endospores per female to harvest time ($P \leq 0.05$). D) Correlation of number of females per root system to degree days was not significant ($P > 0.05$). E) Correlation of number of endospores per root system to degree days was not significant ($P > 0.05$). F) Linear regression of number of endospores per female to degree days ($P \leq 0.05$).

temperature for the development of *P. penetrans* was calculated to be 17 °C (Chen, unpubl.). Therefore, 17 °C was used as the base threshold temperature to calculate degree days. The accumulative degree days above 17 °C were 468.8, 550.8, 627.3, and 684.3 for harvest times of 37, 44, 51, and 58 days, respectively. When plotting the number of females per root system, number of endospores per root system, and number of endospores per female against the accumulative degree days, the relationships were similar to those with harvest times (Fig. A2). The number of females per root system and the number of endospores per root system were not correlated with the accumulated degree days ($P > 0.05$) (Fig. A2D, E), whereas the number of endospores per female correlated with the accumulative degree days ($P \leq 0.05$) (Fig. A2F). The number of endospores per female increased 0.019 million per 100-degree-day increment (Fig. A2F).

The recommended harvest time for the mass production of *P. penetrans* was 7 to 8 weeks after inoculation (Stirling and Wachtel, 1980) or 900 degree days above 10 °C (Sharma and Stirling, 1991). Neither of these studies provided information on the relationship of number of endospores per root system to various harvest times. Thus, the suggested harvest times were rather empirical. The results in the present study suggested that the optimum harvest time might be even longer. Higher numbers of endospores per female could be obtained with a longer plant production time (Fig. A2C, F). Hatz and Dickson (1992) observed that isolate P-20 of *P. penetrans*, which was used in this study, required a longer period of time until mature endospores were first observed. Differences in endospore production over time may be induced by various *P. penetrans* isolates, nematode species, plant hosts, and cultural conditions. The growth of plants in the shade house, which did not allow critical control of temperature, may have resulted in different development of *P. penetrans* than that in studies conducted under constant-temperature conditions. Nevertheless, the results provided information for the

cultivation of *P. penetrans* under conditions that can not be controlled carefully but that may be useful for large scale productions.

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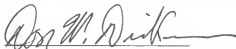
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BIOGRAPHICAL SKETCH

Zhongxiao Chen was born on 15 June 1964, to Mrs. Meihua Wang and Mr. Jiuxian Chen in Beiqian, Yongjia County, Zhejiang Province, the People's Republic of China. Zhongxiao Chen received his elementary and middle school education from the local public schools. He began his undergraduate education in the Plant Protection Department of Zhejiang Agriculture University in November 1979, and received a bachelor's degree in agricultural science in July 1983. He continued his graduate education under the supervision of Professor Defang Fan in entomology at the same department and obtained a Master of Science degree in July 1986. In the next seven years, he was employed in the China National Rice Research Institute, Hangzhou, Zhejiang Province. He was trained in simulation and system analysis of rice production at the International Rice Research Institute, Los Baños, Philippines, 15 January to 30 March 1990. Nematology attracted him to the Entomology and Nematology Department, University of Florida, in January 1994, and he has since worked in the laboratory of Dr. Donald W. Dickson on biological control of plant-parasitic nematodes.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



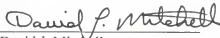
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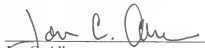
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This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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